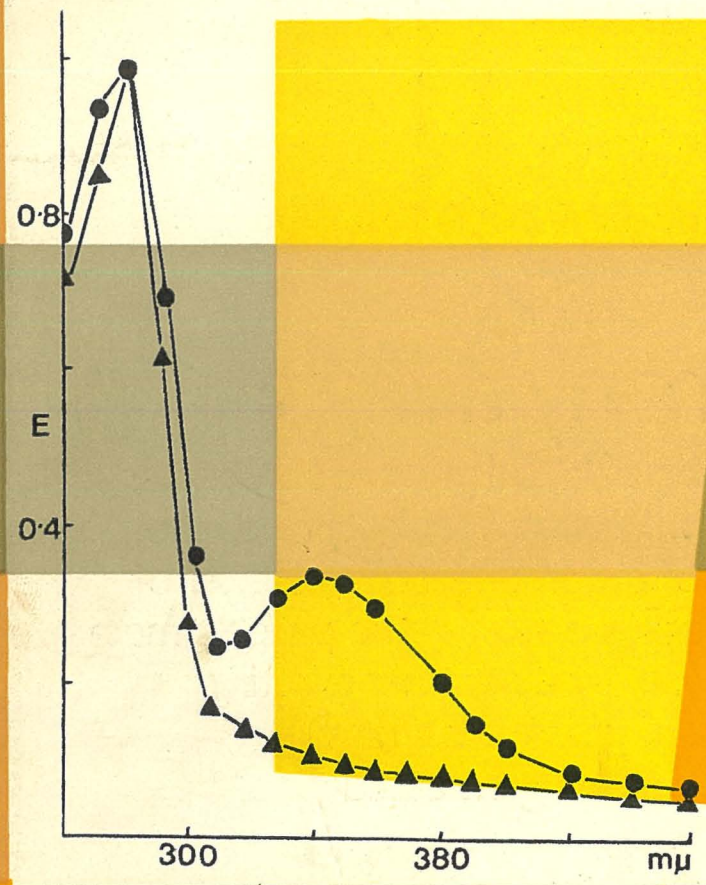
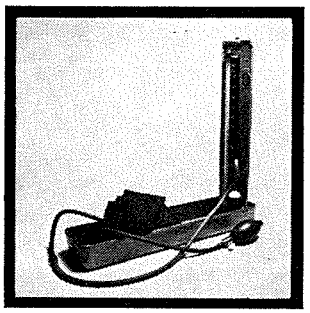
CHESTPIECE



What is progress in the treatment of hypertension?



it is many things:

a firm, rapid lowering of blood pressure in all grades of hypertension.....increased flexibility of control through a shorter duration of action.....fewer of those side effects which trouble some patients more than the disease. By that rather exacting definition, progress is a new hypotensive agent called

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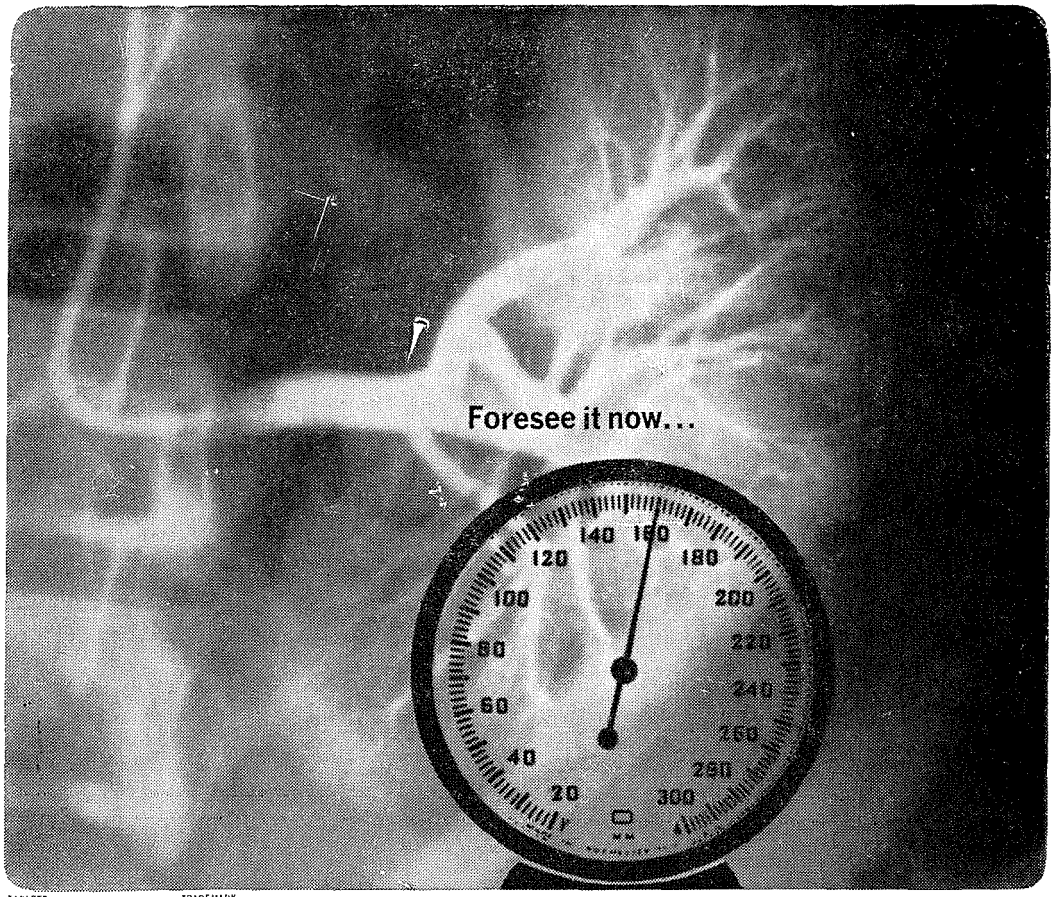

TABLETS TRADEMARK

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1. Brest, A. N.: Hemodynamic response to antihypertensive drug therapy, J.A.M.A.: 192: 127-130, April 15, 1965. (U.S.A.) 2. Onesti, G. et al.: Comparative hemodynamic effects of antihypertensive agents: alpha-methyldopa, pargyline and isocaramidine. Abstracts of the 37th Scientific Session and 18th Annual Meeting, Council on Arteriosclerosis, American Heart Association, October 33:III-135, 1964. (U.S.A.) **Note:** Detailed information is available to physicians on request.

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THE VALUE OF SOYABEAN OIL IN THROMBOSIS AND AS AN ANTI-ARTERIC-SCLEROSE AGENT

The following is reproduced from "NATURE" of January 30, 1960 a U.S.A. medical publication:

DIETARY FATS AND THROMBOSIS

In order to study the influence of the fat content of food on the occurrence of of thrombosis in the arterial and venous system, a group of 133 hospital patients aged 65-90 years were treated with a diet in which butterfat, margarine and lard were replaced by vegetable oils (unhydrogenateed corn oil and soyabean oil). The daily intake of vegetable oils were about 40 gm. or half the total fats. The time of treatment of the 133 patients ranged from 1-17 months (average 4-7 months) representing a total of 52½ years. For comparison, a control group of the same size and composition who received an ordinary hospital diet containing about 80 gm. animal fats (including about 40 gm. butter-fat, margarine and lard) was used. The The occurrence of thrombosis and embolism in both groups was diagnosed clinically and post-mortem.

In the treated group only 4 cases of thrombo-embolism were observed; 1 case of myocardial infarction after 6 days of treatment, 1 case of myocardial infarction after 26 days of treatment, both verified by autopsy; 1 case of little stroke with transient aphasia of a few days' duration occurring after 30 days of treatment, and 1 case of pulmonary embolism after 4 months of treatment in a patient with bronchial cancer (verified by autopsy).

In the control group 15 cases of thrombosis were observed: 2 cases of thrombo-phlebitis (1 case complicated with pulmonary embolism and 1 case with thrombosis in the carotid artery), 2 cases of pulmonary embolism (both verified by autopsy), 5 cases of myocardial infarction (all typical, 2 verified by autopsy), 6 cases of cerebral thrombosis (3 verified by autopsy).

The difference in the occurrence of thrombosis in two patient groups was statistically significant ($t=5.1$) and cannot be interpreted in terms of variations in age, weight and blood pressure in the groups.

Torben Gril
Per From Hansen
Erling Lund

De Gamles By Geriatric Unit,
Copenhagen.
September 23.

The effect of an oil depends on its contents of linoleic and linolenic acids. The two oils which are mainly used as anti-arteric sclerose agents because of their large contents of the two above mentioned acids are Soya and Maize oil. They both contain 50% very often up to 55% of linolenic acid.

Soya has in addition 6-9% of linolenic acid as compared with about 1% in corn oil. The effect of these two acids is not identical but supplementary. It follows that SOYA OIL as an anti-arteric sclerose agent is to be preferred.

THE CHEST-PIECE

JOURNAL OF THE MEDICAL STUDENTS' ASSOCIATION

MALTA MARCH 1968

Inset: Absorption spectrum of Haemocyanin; top: oxygenated; bottom: deoxygenated.

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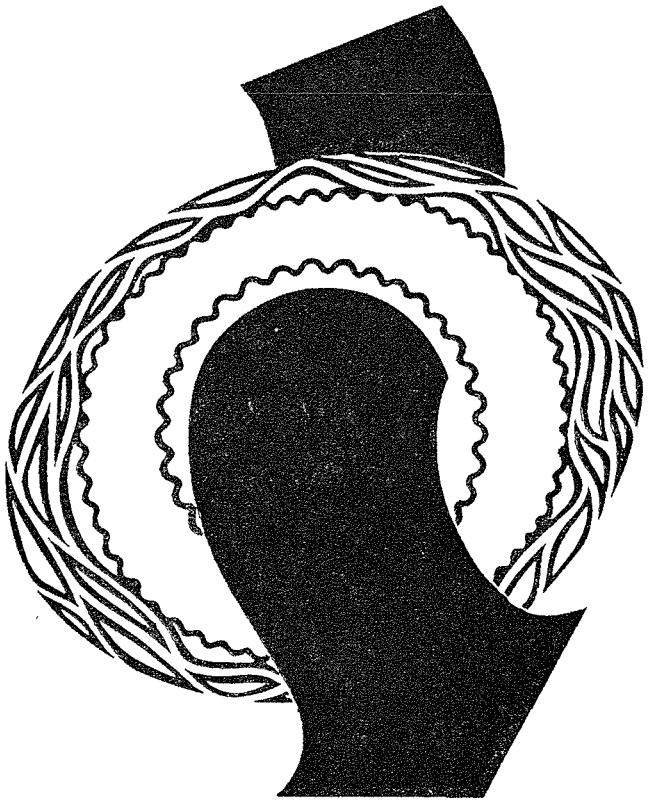

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EDITORIAL

There are times, when no matter how traditionalist one might like to be, the decision must be taken to break with the past and bring oneself up to date.

It was felt that "The Chest-piece" after twenty years of existance needed a more striking cover design and an improvement in the quality of paper and printing if it is to keep up with other medical student journals, and indeed be an expression of the times we live in. There will be those who miss the familiar stethoscope on the cover, but continuity has been sought by keeping the name, even if it has been altered by dropping the "The" and a hyphen.

The cost of printing has soared to unexpected hights, so the recently announced increase in the University subsidy, though inadequate for our needs, was most welcome. Fortunately, financial help has come from the S.P.I.E. and the M.M.S.A. has agreed to cover the deficit. It is planned to publish another edition next October and only an even more generous contribution by advertisers will make this possible.

This journal has never been outstanding for its expression of student opinion but both in this issue and the previous one there have been the early signs of a discontent that is probably not more manifest because of the lack of student solidarity and a seemingly innate fear of retaliation. One of the functions of a student journal is to focus attention on the problems of students and to stimulate their discussion so that they may be solved. Some of the students have complained of being treated like children, and their teachers retort that they deserve this treatment.

The lack of maturity among students is evident from the way we behave at the quarters and more generally, by our attitude and reactions to life in general and our studies in particular. There are several factors that must be considered. A more prominent one is the non-existance of a communal University life, as the majority of us live at home. This familial influence can be unnecessarily inhibiting at times. Secondly, the economic circumstances of the society we live in make it necessary that we should do well in examinations and obtain a remunerative position as soon as possible. Lastly, our years at University have been peppered with a formidable number of examinations which have demanded a photographic memory and have perversely suppressed the ability to think.

The second half of this circle, the Staff, must have a greater share of the blame, as they have the power to change this state of affairs. The clinical course has at present 20 lectures each week, they should last 45 minutes, but some are prolongued unbearably. Only a few are illustrated with slides and other aids. In contrast there are only 5 tutorial-cum-demonstrations, most of which are instructive and enjoyable. The remaining 12 hours per week dedicated to the wards are completely insufficient for a profession that is mainly practical.

When it comes to examinations, the situation shows no improvement. The final course has four major subjects and six minor ones, each with separate examinations. The incorporation of the minor subjects into the major ones would ease the burden on the students, especially as too much detail is expected of our answers in the written parts.

When the University authorities give us more freedom to learn about our subject by the methods we choose and when more free time is provided to take part in activities outside the curriculum, they will probably realise that we can be responsible and trustworthy.

ACKNOWLEDGEMENTS

We are very grateful to Norbert Attard for the excellent cover design and the allied art-work on which he has been working for the past two months. Our thanks are also due to Bernard Anastasi and Anton Bencini for art-work of a very high standard in connection with illustrated articles. Mr. J. Xerri has been most kind and cooperative in supplying the photographic material. Finally, a sizeable part of the credit for this issue of our journal goes to Mr. Gnagi and the staff at St. Joseph's Institute Press for invaluable help and encouragement.

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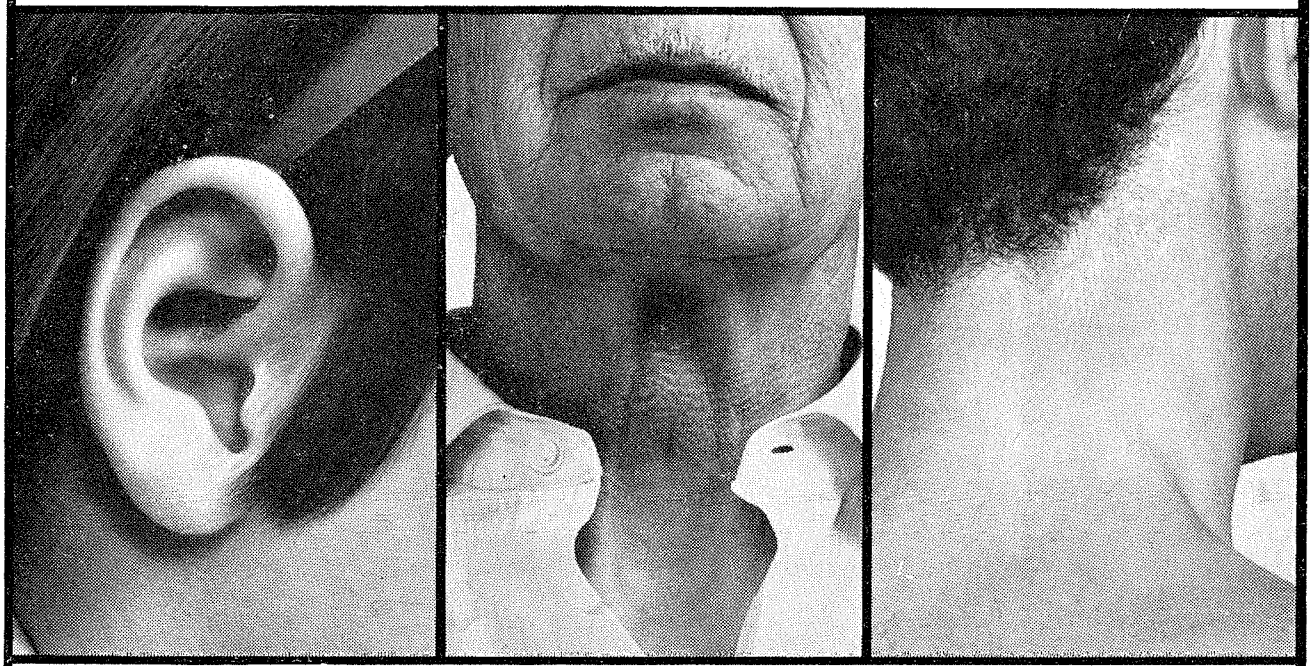

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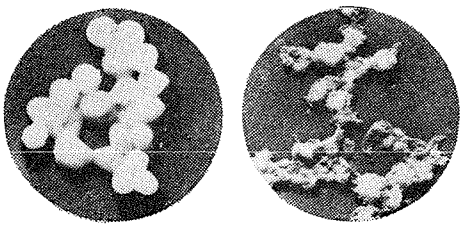

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THE COPPER PROTEINS

E. J. WOOD, M.A. (Oxon.)

Department of Physiology and Biochemistry, Royal University of Malta

INTRODUCTION

The presence of copper in plant and animal tissues has been known since 1816 and 1833 respectively(1,2), but until the 1920's it was usually assumed to be there by accident. Nevertheless in 1847 HARLESS (3) had detected copper in snails and had shown that it existed in combination with the blood proteins in these invertebrates. Between 1920 and 1930 nutritional studies established that copper was an essential trace metal and was necessary for haemopoiesis. Since that time a large number of copper-containing proteins have been isolated from animal, plant and microbial sources. Some of these proteins have well defined functions such as enzyme activity or the ability to transport oxygen, while others have no known function.

The nutritional aspects of copper have been reviewed by UNDERWOOD(4), but it is convenient to summarize present knowledge of copper deficiency and copper toxicity here. The copper content of the whole human body is 100-150 mg., but infants have two to three times more copper per unit of body weight. The majority of the body copper is found in liver, heart, kidney, hair, brain, bones and muscle, while spleen, lungs, and especially endocrine glands contain relatively smaller amounts. The liver appears to act as the main storage organ, and its copper content may be taken as a fairly reliable index of copper status.

Copper is relatively poorly absorbed. Depending on the other constituents of the diet, 75-90% of dietary copper (as ^{64}Cu) appears in the faeces in 3-5 days along with a little in the urine. The kidneys do not appear to be an important route of copper excretion, but on the other hand, the bile tends to be relatively rich in copper. There is no good estimate of the daily dietary requirement for copper; most authorities quote 1-2 mg./day.

Copper deficiency in animals manifests itself in a number of ways: anaemia, depressed growth, bone disorders, depigmentation of hair, fibrosis of the myocardium, and diarrhoea have all been recorded, but not all in the same species at the same time. Copper has long been known to be toxic, both acutely and chronically, but again no reliable figures are available for the effects and tolerated doses in humans. In animals about 4-10 times the normal requirement over a period appears to do no harm, and in rats, toxic effects are not seen until about 100 times the normal requirement is given. Excessive ingestion of copper in animals leads to passive accumulation in the tissues, especially the liver. Up to a certain point this is tolerated without apparent ill effects, but beyond this level there may occur a catastrophic liberation of a high proportion of the copper into the blood. Haemolysis, jaundice, and usually death ensue.

A number of laboratories are engaged in the study of the copper proteins. Besides satisfying the fundamental curiosity of biochemists there is the hope that biochemical studies may throw some light on copper deficiency and copper toxicity, as well as on the problem of Wilson's disease (see below).

For the sake of convenience the copper proteins will be discussed in three sections: (1) mammalian copper proteins; (2) haemocyanins; and (3) plant and microbial copper proteins. Some of the properties of the copper proteins are set out in Table I. This list is not exhaustive: the copper proteins continue to be discovered, while on the other hand, in one or two cases, a protein thought to be a copper protein has turned out to be merely contaminated with copper. The table does include all those copper proteins that have been studied in any detail.

THE MAMMALIAN COPPER PROTEINS

Ceruloplasmin. Normal plasma contains a copper protein which was isolated and crystallized by HOLMBERG and LAUREL(5) in 1947-8. It was called ceruloplasmin because of its blue colour. It has a molecular weight of 160,000 and contains 8 atoms of copper per molecule, and it also has oxidase activity. In electrophoresis it moves with the alpha-globulins, and almost all of the copper in mammalian plasma is in the form of ceruloplasmin. Interest in ceruloplasmin was stimulated when in 1952(6) it was shown that ceruloplasmin was deficient or even absent from the plasma of patients with Wilson's disease(7,8,9). In 1911 WILSON(10) described: "... progressive lenticular degeneration, a familial nervous disease associated with cirrhosis of

TABLE I

Properties of some of the copper proteins

Protein	Source	M.Wt.	%Cu	State of Cu	Cu atoms /mol.	Colour (abs. max.)
Ceruloplasmin	serum	160,000	0.34	$\frac{1}{2}$Cu+ /$\frac{1}{2}$Cu++	8	blue (610mu)
Erythrocuprein	rbc's	33,500	0.38	Cu++	2	blue-green (655mu)
Haemocuprein	rbc's, liver	30,000	0.35	Cu++?	2	blue
Hepatocuprein	liver	30-40,000	0.35	Cu++?	2	blue-green (660mu)
Cerebrocuprein	brain	35,000	0.30	Cu++?	2	blue-green (660mu)
Amine oxidase	liver mitochondria	250,000	0.05-0.1	Cu++	4	colourless/ pink
Cytochrome oxidase	mitochondria	?	?	Cu++?	2?	(830mu)
Uricase	liver	120,000	0.06	?	1	?
Dopamine beta-hydroxylase	adrenals	290,000	0.10	Cu++	4-7	colourless
Haemocyanin	arthropods	0.4-1x10^6	0.15-0.19	Cu+	ca.20	blue (570mu)
Haemocyanin	molluscs	2-9x10^6	0.23-0.26	Cu+	200-400	blue (570mu)
Tyrosinase	fungi	130,000	0.20	Cu+	4	colourless
Laccase	lac tree	130,000	0.33	Cu+/Cu++	4	blue (615mu)
Ascorbate oxidase	plants, bacteria	140,000	0.34	6Cu+/2Cu++	8	blue (608mu)
Galactose oxidase	fungi	75,000	0.09	Cu++	1	pink-yellow
Azurin	bacteria	14,600	0.45	Cu++	1	blue (625mu)
Plastocyanin	chloroplast	21,000	0.58	Cu++	2	blue (597mu)
Stellacyanin	lac tree	20,000	?	?	1	blue (604mu)

the liver...": the name 'hepatolenticular degeneration' is still commonly used for the disease. Two years later it was reported(11) that there was an excess of copper in the liver in this disease, and later it was shown that this applied to the brain too. In 1948 it was suggested(12) that the sulphydryl chelating agent British Anti-Lewisite (BAL) might be used therapeutically in Wilson's disease.

Ceruloplasmin does not bind copper irreversibly, since the copper atoms can be exchanged for radioactive copper under suitable conditions. It is also possible to prepare copper-free, or apo-ceruloplasmin, and this can be reconstituted by adding copper, to give native ceruloplasmin once again. In fact the eight copper atoms in a molecule of ceruloplasmin are not identical: four of them appear to be loosely bound (exchangable with ^{64}Cu), while four are more tightly bound (dialysable only after chymotryptic digestion). Probably four are Cu++ and four are Cu+ (see ref. 13): nevertheless the Cu+ atoms of ceruloplasmin do not bind oxygen as do those of haemycyanin.

Perhaps the most notable feature of ceruloplasmin is its oxidase activity towards a variety of polyamines and polyphenols, and possibly ascorbic acid. However, despite a great deal of experimental work on the enzymic properties of ceruloplasmin, the physiological significance of this activity remains completely obscure. Ceruloplasmin could represent a copper transport system in the plasma which just by chance happens to have oxidase activity. However, some patients with Wilson's disease, controlled with penicillamine, appear to lead a normal life with no detectable copper and no detectable ceruloplasmin in the serum. On the other hand, though ceruloplasmin has a number of oxidase activities, the substrates for oxidation so far studied can occur rarely, if ever, in the plasma, with the exception of ascorbic acid. The range of compounds that can be oxidised in presence of ceruloplasmin is very wide. **p**-Phenylenediamine (PPD) is a good substrate, and a number of other, similar compounds have been compared with it. In order, with respect to ease of oxidation, some of these compounds are: PPD; N,N-dimethyl-PPD; N-methyl-PPD; p-aminophenol; hydroquinone; N-N-dimethyl-MPD; MPD (MPD=**m**-phenylenediamine). The reaction sequence that has been suggested for these oxidations is complex and involves free radical formation and conversion of the Cu++ of ceruloplasmin to Cu+. Finally the Cu+ is reoxidised by molecular oxygen to Cu++. Other compounds whose oxidation is catalysed by ceruloplasmin include several reducing agents, hydrosulphite, hydroxylamine, thioglycolate, and hexacyanoferrate.

The significance of ceruloplasmin as an ascorbic acid oxidase is both obscure and controversial. It is fairly well established that ceruloplasmin can catalyse ascorbate oxidation, but the demonstration is difficult for two reasons. Firstly the activity is low in comparison with true ascorbate oxidases from plants, and secondly free cupric ions (with which preparations of ceruloplasmin may be contaminated) also catalyse the oxidation of ascorbate; indeed, under certain conditions they do so more efficiently than does ceruloplasmin (see discussion in ref. 14). Such ascorbate oxidation in the serum might in any case be considered biologically wasteful, since the energy released presumably could not be used for phosphorylation. On the other hand, it has been shown(15) that the product of ascorbate oxidation, dehydroascorbate, penetrates brain and eye tissues, and erythrocytes more rapidly than ascorbate. This might conceivably represent some sort of transport system, but set against this must be the very low ascorbate oxidase activity of ceruloplasmin, despite its relatively high concentration in serum.

We are left, then, with only a very vague idea of the function of ceruloplasmin. (It might be that ceruloplasmin is an evolutionary relic, in the sense that most, if not all, animals have a copper protein in the blood, and in some of them (molluscs and arthropods) this has evolved into a respiratory carrier for oxygen (haemocyanin, see below). Furthermore, some recent work has suggested that lack of ceruloplasmin may not be the primary lesion in Wilson's disease. The original hypothesis(16) was reasonable: failure to sythesize the protein led to the plasma copper remaining diffusible, with the result that it entered the tissues and accumulated. In liver, excess copper produced necrosis and fibrosis, in kidney, deficient tubular reabsorption, in brain, degeneration of the basal ganglia and consequently the characteristic motor disturbances, and finally in the eye, the pigment rings which are diagnostic of the disease. Unfortunately there is poor correlation between ceruloplasmin concentration and the duration and severity of Wilson's disease(16), and, indeed, in a few cases, the blood ceruloplasmin level appears to be almost normal(17,18).

Wilson's disease is inherited in a recessive manner, and the heterozygotes can be identified by the fact that, like the homozygotes, they show delayed incorporation of copper into ceruloplasmin(19). It was a possibility therefore that the ceruloplasmin present in some patients with Wilson's disease was structurally abnormal and could not bind copper properly. In a recent study however(20), tryptic peptide maps were made with ceruloplasmin isolated

from an individual with Wilson's disease whose blood ceruloplasmin level was close to the normal. The peptide maps were normal, which would seem to rule out the synthesis of an abnormal protein in this genetic trait. Thus it may be that we will have to look to the other copper proteins of mammals for an explanation of the defect in Wilson's disease.

Other Mammalian Copper Proteins. Our knowledge of the small copper proteins of the body, namely erythrocuprein, haemocuprein, hepatocuprein, and cerebrocuprein, goes little farther than what is summarised in Table I. As is seen, these are all similar in copper content, colour, and size, and in addition they are all characterised by a lower than normal absorption in the ultraviolet (280mu). In this latter respect they are abnormal compared with almost all other proteins in having a very low content of aromatic amino acids. For example, amino acid analysis of highly purified human erythrocuprein showed that tyrosine and tryptophan were absent(21). That these soluble tissue copper proteins are nevertheless different proteins is shown by their distinct chromatographic behaviour and by their immunological properties.

These copper proteins account for a considerable proportion of the tissue copper. Cerebrocuprein, hepatocuprein, and erythrocuprein each account for about 60% of the total tissue copper of brain, liver, and erythrocytes respectively. These proteins have no known function, nor has any enzyme activity been attributed to them so far. They may be present as copper storage depots and in addition may be important in picking up any free copper in the cell. For a number of reasons free copper cannot be allowed to accumulate in the tissues of a normal individual. While some enzymes need copper for activity, others are strongly inhibited by copper ions. Copper ions form fairly stable chelates with amino acids and could therefore interfere with the process of protein synthesis. In addition copper ions can combine non-specifically with carboxyl groups and sulphydryl groups of proteins, possibly altering the overall properties of the proteins considerably. No doubt in copper toxicity we are observing such derangement of these processes.

PORTER(22) has recently partially purified a new tissue copper protein from the liver mitochondria of newly born infants, which he has called "neonatal hepatic mitochondrocuprein". This contained more than 3% copper, and was distinct from the other liver copper proteins including cytochrome oxidase, and probably performs a storage function for copper in the immature animal analogus to that of ferritin for iron storage. It is interesting that the liver of the newly born infant contains a much higher concentration of copper than other tissue, and also that the mitochondrial fraction of new born liver may contain up to 30 times as much copper as the mitochondrial fraction from adult liver. In fact a newly born infant is similar to a patient with Wilson's disease, in that he has a high liver copper and also lacks the normal amount of ceruloplasmin.

Cytochrome oxidase(23,24), the terminal electron acceptor of the electron transport chain, is a haem-copper-protein, is almost universal in cells, and is located in the mitochondria. It seems probable that the unit of cytochrome oxidase contains one cytochrome **a** and one cytochrome **a3**, and that each cytochrome is associated with one copper atom. Kinetic studies indicate that the cytochromes as well as the copper atoms participate as electron carriers in the electron transport chain. Such kinetic experiments do however require very careful interpretation. That cytochrome oxidase is an integral component of the mitochondrion makes its isolation and purification all the more difficult. As a result there has been much discussion and controversy, not only as to whether preparations are contaminated with extraneous copper, but also whether the copper does in fact participate in electron transport.

Some of the amine oxidases are somewhat better characterised, and these, like cytochrome oxidase, are widely distributed in animals, plants, and bacteria(25). The general reaction that they catalyse is:

$$R\text{-}CH_2NH_2 + O_2 + H_2O \longrightarrow R\text{-}CHO + NH_3 + H_2O_2.$$

Amine oxidases have been classified into mono- and di-amine oxidases, but recently this classification has been criticised on the basis that "diamine oxidase" acts on many monoamines and *vice versa*(26). It seems probable that all amine oxidases will turn out to be copper proteins(25) but another cofactor is always present, either pyridoxal phospate or a flavin.

The so-called "diamine oxidase" from pig kidney proved to be the same protein as histaminase(27), in other words the one protein attacks **both** diamines and histamine. In a similar way "monoamine oxidase" acts on both alkylamines and arylamines(28). The function of monoamine oxidase seems to be breakdown of amines of high biological activity, both naturally occuring and pharmacological. The most important substrates are tryptamine derivatives (5-hydroxy-tryptamine) and catechol amines (adrenaline, dopamine). Diamine oxidase on the other hand is concerned with histamine breakdown and with breakdown of various biologically active diamines.

Unlike most of the other copper proteins, amine oxidases are either colourless (hog kidney diamine oxidase) or pink (monoamine oxidases from various sources). Nevertheless the copper appears to be in the cupric state, and in the case of histaminase at least, is probably bound to the pyridoxal prosthetic group(28). Clearly a great deal of work remains to be done before our knowledge of the structure and mechanism of action of both cytochrome oxidase and amine oxidases is anywhere near complete.

THE HAEMOCYANINS

Haemocyanins are high molecular weight copper proteins which are found in the blood of some, but not all molluscs and arthropods (29,30). They have the property, like haemoglobins, of combining reversibly with oxygen, but unlike the haemoglobins they are dissolved directly in the blood or or haemolymph rather than being held in the corpuscles. In most of the specimens of haemolymph that have been examined, haemocyanin has been found to account for 90-98% of the protein present.

TABLE II
Comparison of Haemoglobin and Haemocyanin

Property	Haemoglobin	Haemocyanin
Metal	Iron (Fe++)	Copper (Cu+)
Porphyrin	yes	no: copper bound directly to protein
M. Wt.	67,000	400,000-10,000,000
Colour	red	blue
Structure	2 pairs identical subunits	half, and eighth-to-twelfth mols. may be obtained
Occurence	erythrocytes	dissolved in blood

Since haemocyanins and and haemoglobins have the same function, that is, oxygen transport in the blood, it is instructive to compare the two proteins, As Table Two shows, apart from being oxygen-carrying metallo-proteins, they could hardly be more different—a striking example of convergent evolution presumably. However it should be mentioned that some investigators have thrown doubt on this oxygen-carrying function of haemocyanins. It is pointed out(31) that in some crustaceans the haemocyanin content of the blood at certain moulting stages is lowered to levels such that the function of the pigment is completely acessory. Furthermore, PILSON(32) found a great variation in the haemocyanin content of the molusc, **Haliotis** which was unrelated to weight, sex, or reproductive activity. Thus it is possible that haemocyanins represent some sort of food or copper reserve which just happens to be capable of carrying oxygen. It is clear that many questions concerning haemocyanin remain to be resolved. Apart from doubts about the function of the protein, there is in addition very little knowledge about the synthesis of the protein or the mode of attachment of the copper. It is interesting that haemocyanins have been reported to possess enzymic activity in addition to their oxygen-carrying ability. The activities that have been observed are catalase, polyphenoloxidase, and lipoxidase(33).

Several of the properties of haemocyanins have been examined in great detail. Particular attention has been given to the oxygen-carrying ability, the subunit structure and the mode of attachment of the copper atoms. It may be hoped that information obtained about haemocyanins will be of use in relation to the study of other copper proteins.

It has long been known that during the oxygenation of haemocyanin, when the colour of the compound changes from colourless to deep blue, one molecule of oxygen is bound for every two atoms of copper. The oxygen dissociation curves for haemocyanin are largely homologous with those of other respiratory pigments (though, of course, they differ in detail), and have shapes from hyperbolic to sigmoid. The shape of the curve depends on a number of factors including the stage of purification, the pH, and the presence or absence of certain ions, especially calcium and magnesium. There has been some controversy as to whether there is any interaction between the oxygen-binding sites on the same molecule. Recent reports would seem to indicate that there is a negative interaction. Since in the largest molecular weight haemocyanins there are several hundred copper atoms, and since the molecules are capable of splitting into subunits under certain conditions (see below), any interaction must surely be very complex indeed. It has been found that thiocyanate and thiourea(34), and more recently ethyl isocyanide(35) cause the reversible expulsion of oxygen from haemocyanin. Further study of such reactions may lead to a better understanding of the oxygenation reaction.

The subunit structure of haemocyanins has long attracted the attention of the biochemists and biophysicists. Early ultra-centrifuge studies on the molecular weight of haemocyanins revealed that, depending on the conditions of pH and ionic composition, the molecule could undergo dissociation into subunits. Generally,

the components of the highest molecular weight always exist in the pH region around the isoelectric point. On either side of this region smaller units are formed, but units of different sizes may co-exist at a particular pH. Thus in the case of the haemocyanin from the snail, **Helix pomatia,** over the pH range 4.7-7.0 (the stability region) a single high molecular weight species is present, from pH 4.6-3.6, and from pH 7.4-8.1, whole and half molecules are present, and outside these regions eighth or tenth molecules are formed. It seems fairly certain that in the blood of marine invertebrates, which has a pH of 8.2-8.3, the haemocyanin molecules are undissociated, due to the presence of Ca++ and Mg++ in the haemolymph. These ions have the property of extending the stability region of the whole molecules, so that to this extent the process of dissociation is probably un-physiological. One may speculate that these animals, instead of evolving blood corpuscles to contain the respiratory pigment, have evolved giant molecules which have somewhat similar properties. For example they would not be able to diffuse out of the blood system into the tissues. It has been possible to demonstrate something of the subunit structure in the electron microscope. VAN BRUGGEN and his coworkers(36) showed that the molecule of **H. pomatia** haemocyanin at pH 6.0 was a cylinder of length 335A and diameter 300A, and had a five-fold axis. This five-fold axis may explain why one-tenth molecules can be obtained under certain conditions.

Perhaps the most interesting feature of haemocyanin is its copper atoms, which most workers consider to be in the univalent or cuprous state in both oxygenated and deoxygenated haemocyanin. The reasons for believing this to be the case come from a number of sources. For example, the absorption spectrum of haemocyanins is not like that of copper proteins known to contain cupric copper, most, if not all of which absorb at 610mu. Furthermore copper can be removedf rom haemocyanin with KCN to give a colourless protein, and the copper can then be restored by the addition of cuprous, not of cupric, compounds. However, this evidence is not completely satisfactory, and some investigators have suggested that in the oxygenated molecule the copper is half cuprous and half cupric(37).

Related to the question of valency of the copper is the problem of how the copper is joined to the protein. Since all attempts to demonstrate the presence of a porphyrin or similar cofactor have failed, it is assumed that the copper is linked directly to the amino acid side chains of the protein. Nevertheless, at present it is impossible to state which of the amino acids in the protein bind the copper atoms. The copper is certainly very tightly bound, and is only removed by cyanide or by agents which cause denaturation of the protein molecule. Many compounds capable of chelating copper have been tried, but, with the exception of cyanide, none has removed a significant proportion of the copper. The residues most likely to be implicated in the binding of copper would seem to be cysteine (-SH) and histidine (imidazole). There is little evidence to support the idea of copper binding by cysteine sulphydryl groups, while evidence from titration curves(33) and from photooxidation experiments(38) suggest that histidine residues are involved, though not necessarily exclusively, in copper binding. It may be said at this point that little is known of the mode of binding of copper in **any** copper protein. Certainly evidence obtained with haemocyanin may provide valuable clues about copper binding in other copper proteins. If only for this reason, the investigation of haemocyanins represents a very fruitful field of study.

THE PLANT AND BACTERIAL COPPER PROTEINS

The copper proteins of plant and microbial origin comprise a group of enzymes, tyrosinase, ascorbate oxidase, laccase, and galactose oxidase, a component of the photoynthetic system, plastocyanin, along with several proteins of unknown function, such as stellacyanin from the Japanese lac tree, the blue copper protein from mung bean seedlings, and azurin from bacteria. No doubt many more such proteins will be discovered. It is proposed to limit the present discussion to the first group, the plant copper enzymes, since these have been most studied. Details of some of the other proteins appear in Table I.

Tyrosinase. This protein has long been known to contain copper(39). It can catalyse two different aerobic oxidation reactions: (a) hydroxylation of tyrosine ("cresolase activity"), and (b) dehydrogenation of a number of **o**-dihydric phenols or catechols ("catecholase activity"). Because of this latter activity it has also been called polyphenol oxidase. In fact, tyrosinase does not only occur in plants and fungi. The tyrosinase that has been studied the most is that from mushrooms, and it is for this reason **only** that it is included in this section. Tyrosinases are also known from insects and from mammals. Tyrosinase is the enzyme responsible for the formation of pigments, melanins, in animals throughout the phylogenetic scale. These include the pigments of skin, eyes, and feathers: thus tyrosinase has been isolated from skin and from melanomatous tissue. Tyrosinase is clearly

a very important enzyme—its action is connected with both medical and social problems.

The relationship between the two types of activity of the enzyme is the subject for considerable controversy. Some workers consider that both activities are contained within the same protein molecule, whereas others think that it is the products of catecholase action that are responsible for the hydroxylation activity(40). A further suggestion worthy of consideration is that the enzyme is composed of subunits, some having largely cresolase activity and some having largely catecholase activity. The resolution of this controversy must await further work with the enzyme. Nevertheless, depending on the source and isolation procedure used, the enzyme may turn out to have either a high ratio of catecholase to cresolase activity, or a low one.

Tyrosinase is unusual among the copper proteins in being colourless. Examination of the spectrum reveals a strong absorption of 280mu due to the high content of aromatic amino acids, and a weak absorption at around 340mu.

TABLE III
Comparison of Spectral Properties of some Copper Proteins

Protein	State of Cu	280mu	340mu	570mu	610mu
Tyrosinase	Cu+	+++	+/—	—	—
Haemocyanin	Cu+	++	—	—	—
Oxyhaemocyanin	Cu+/Cu++?	++	+	+	—
Ceruloplasmin	Cu+/Cu++	++	+	—	+
Erythrocuprein	Cu++	+	—	—	+

Furthermore there is no difference between the absorption spectrum of the enzyme and that of the copper-free apo-enzyme. If the spectrum of tyrosinase is compared with the spectra of other copper proteins (Table III) it is seen that only proteins containing Cu++ appear to absorb in the 610mu region of the spectrum. Tyrosinase seems to contain only Cu+, but it is important to perform the valency determination on freshly prepared material, since on storage, there is partial oxidation of the copper with a corresponding loss in enzymic activity(41).

Ascorbate Oxidase. This enzyme, isolated from many plant tissues (apple, potato, spinach), catalyses the aerobic oxidation of ascorbic acid to dehyroascorbic acid(42). It has never been detected in animal tissues. Solutions of the enzyme are blue, but the colour is bleached when ascorbate is added, oxygen is absorbed, and then, when all the ascorbate has been oxidised, the colour returns. The copper-free apoenzyme prepared by dialysis against cyanide is also colourless. The active copper enzyme can be regenerated by the addition of Cu+ but not of Cu++ salts. Experiments indicate that the copper exists in the native enzyme in a mixed valence state where Cu++:Cu+ = 3:1. Thus the copper in the enzyme seems to be somewhat similar to that in ceruloplasmin, and it is likely that only the Cu++ is involved in enzymic activity. (It will be remembered that ceruloplasmin also shows ascorbate oxidase activity).

It is interesting that removal of the copper with cyanide leads to an apo-enzyme containing 10 free sulphydryl groups, whereas the native enzyme contains no sulphydryl groups. This might seem to indicate that the copper is bound to sulphydryl groups. However, this does not appear to be the case, since if the apo-enzyme is treated with p-chloromercuribenzoate, which blocks sulphydryl groups, the enzyme activity and copper may still be restored. This conclusion is strengthened by the finding that if the native enzyme is denatured (i.e. the protein chain is opened out) 10-12 sulphydryl groups are revealed. It looks therefore as though these sulphydryl groups have nothing to do with the binding of copper, but instead have some part to play in stabilising the structure of the protein. It has been suggested that the copper might be bound to histidine + alpha-amino groups, and/or histidine + carboxyl groups in the protein.

The function of the ascorbate oxidase in plants is uncertain. It is possible that there is a multienzyme system in plant respiration involving glutathione reductase, dehydroascorbate reductase, and ascorbate oxidase(43) (Fig. 1). It has long been known that plant tissues contain glutathione reductase and dehydroascorbate reductase, but the latter is absent from the mitochondria. From the results obtained with pea seedlings(43)it it seemed possible that about 25% of the total respiration might proceed by this route.

Laccase. If thin layers of the latex from the Japanese lac tree, **Rhus vernicifera,** are exposed to moist air they darken and dry to give a lustrous translucent material which forms the basis of a furniture finish. Investigation of the latex(44,45) led to the isolation of a copper enzyme, laccase, which catalyses the oxidation of certain hydroquinones and semiquinones. The semiquinones then react non-enzymically to

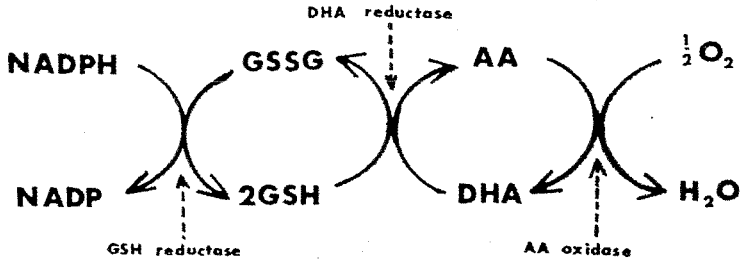

Fig. 1. Possible terminal oxidative pathway in pea seedlings. GSH, GSSG are reduced and oxidised glutathione, AA, DHA are ascorbic acid and dehydro-ascorbic acid, respectively.

produce the corresponding quinones. The semi-quinone formation is the result of a one electron transfer from the substrate to copper of laccase to produce cuprous copper which is then re-oxidised to cupric copper by molecular oxygen.

Laccase has since been found in many plant tissues. It is essentially an extracellular enzyme, and the darkening and hardening of lac tree latex is a result of the action of the enzyme, in the presence of oxygen, on "laccol", which is not a single compound, but a whole group of similar compounds. These compounds may be regarded as the true substrates of the enzyme. The basic structural feature of the whole molecule is that it is a 1,2-diphenol with a long unsaturated hydrocarbon chain at position 3 of the phenol ring. Nevertheless, laccase also catalyses oxidation of a number of simpler aryl diphenols and diamines, as well as coniferyl alcohol, which is a percursor of the lignin of woody plants. Thus laccase may be connected with the process of wood formation, and probably also with wound repair in plants. It is interesting that the wood rotting fungus, **Polyporus versicolor**, produces laccase and uses it to break down the lignin of woody tissue.

Galactose Oxidase. This is a copper enzyme produced by fungi, and has been isolated from **Polyporus circinatus.** The reaction that it catalyses is the oxidation of **D**-galactose at carbon 6 to give D-galactohexodiadose(13). The enzyme is one of the few copper proteins whose copper is removed by strong chelating agents or by hydrogen sulphide.

SOME CONCLUSIONS

In the average text-book of biochemistry one may find perhaps half a page out of, say, the thousand pages in the book devoted to copper proteins. On the other hand, wherever one looks in living organisms one finds copper proteins of one sort or another. It is not the purpose of the present author to criticise this state of affairs, but rather to redress the balance by showing what an interesting group of compounds the copper proteins form, and what a wealth of material there is for study.

No attempt will be made to summarise the foregoing discussion, because in itself it represents but a summary of a considerable amount of work that has been performed. Instead, one or two conclusions can be drawn which represent general principles in the field of copper proteins:

(1) Copper though itself toxic, is found as a component of proteins from almost all life forms.

(2) Copper proteins are almost always blue in colour if they contain cupric copper, but colourless if they contain cuprous copper. Intermediate cases are however encountered which make it difficult to apply this rule to all copper proteins.

(3) Copper proteins are nearly always associated with oxygen in some way, either as oxygen carriers or as oxidase enzymes in which molecular oxygen participates in the enzyme reaction. This is presumably a reflection of the ability of copper atoms to change from Cu++ to Cu+ and back again.

(4) Though the sulphydryl groups of cysteine residues in proteins would appear to be likely candidates for binding copper, only in one case (galactose oxidase) has this been demonstrated, and in most other cases there is actually evidence **against** the participation of sulphyryl groups.

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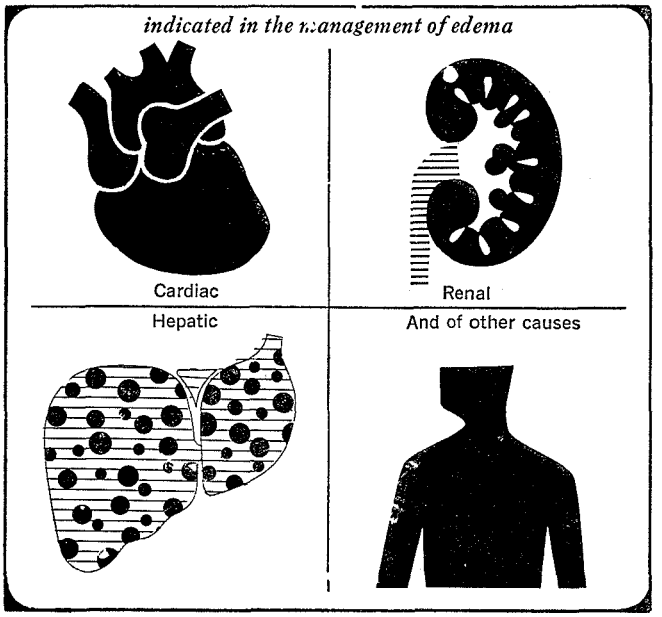

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HEADING TOWARDS THE HIPPOCRATIC OATH

ANTON J. BISAZZA

We are expected to know how to perform a Caesarean Section, how to differentiate between Ankylosing Spondylitis and Polyarteritis Nodosa, how to do a rectal examination without making our patient whimper, how to cook up Lowenstein—Jensen's medium, how to distinguish between the various forms of Malaria, how to plan a diet for a patient with pyloric obstruction, how to set up a drip, how to apply a figure-of-eight bandage, and how to go about removing a glioblastoma multiforme. That's us, the long-suffering, much misunderstood and often over-rated medical students.

We first enter University as pimply, expectant school-leavers who believe that this is where life is really enacted as it should be. We are greeted by a beaming Professor of Physics, a gentle-looking Professor of Chemistry, a wise-looking Professor of Biology, a historical-looking Professor of Philosophy and a cynical, very-much-aware-of-how-ridiculous-all-this-is Mathematics lecturer. This is the preliminary course of Medicine and Surgery, and is designed at preparing the future doctor with a firm grounding in the vascular system of the buttercup and cockroach, the large-scale industrial preparation of steel, the method of learning how to be patient by using a stop-watch and a 10 ft. long pendulum, the history of Metaphysics in the light of Freudian philosophy, and as if all this were not bewildering enough for a "keen medical student", a good measure of Calculus. To this very day I still can't see why all this was taught to us and why it had to be instrumental in causing many bona fide students to give it all up as a bad job.

Two years later and before we know it, we're third year medical students convinced that "this is where we come face to face with our profession at last". Our spirits, however, are soon dampened by an immensely overpowering and greatly amused professor who tells us our only chance of passing Anatomy lies in reading "Aids to Anatomy" while standing in a cinema queue. We turn to our Professor of Physiology for consolation, and here at last is a subject that appears amenable to reason, and not merely memory-work. This state of affairs only lasts until we meet our Bacteriology lecturer. Much to our dismay we learn that success in this subject is forthcoming only if we manage to remember what brand of frog's legs Pasteur preferred for his Sunday dinner. We get down to brass tacks and somehow, in the short space of 22 months we learn something about the human body, its functions and its pests (be they indole-positive or negative). It is at this stage that most of us are palpating for the head of the fibula while at the cinema with our girl; our celibate colleagues on their part will be feeling for the supraclavicular lymph modes on their protesting but fascinated grandmother. By hook or by crook (be the latter in the form of either Coryn. Diphtheriae or of the branches of the sacral plexus) we make it to S.L.H. Oh, boy talk of fun and frolics!

From the relative security and comfort of the basement at the Evans we find ourselves at a glaring antiseptic-looking building bearing the legend "Royal University of Malta Medical School' 'in dramatic bronze lettering. One of my fellow students felt so proud of this achievement that he got drunk, fell down the stairs and ended up in the receiving end of M S 2 for a couple of days. In a matter of days we forget all our Anatomy and Physiology and are gamely struggling with such fantastic terms as idiopathic cephalalgia, erythromelalgia, cachexia ovarica and tic doloreaux. Fortunately all our demonstrators flatter us and help increase our self-esteem merely by assuming that in a matter of 48 hours we have learnt all about these conditions, all of which seem to rear their ugly heads just when we have started our final course. It is at this stage of our clinical career that our keener colleagues begin to assume they are doctors. (A particularly enthusiastic one told us not to refer to him as a student in front of patients). Something else happens, however, and this is that we all start realizing how silly the whole thing is. For example, each one of us plans our own study programme, only to have it all shattered by an order to take end of term tests in all subjects, with the unpleasant consequence of bringing a professor's wrath onto our heads if we don't comply. We are told that this is for our own good, but we all feel that a certain amount of liberty is to be given to us if we are to become responsible self-confident doctors. Another unpleasant realisation during our final course is that each and every specialist who teaches us expects us to know everything about his subject. I am convinced and state categorically that as intending G.P.'s lots of our time is being wasted on teaching us and expecting us to know, how to manage a case of eclampsia, how to diagnose the site of a cerebral tumour and how to perform a splenectomy. It would be far better for all parties concerned if more time were spent teaching us how to do practical things like suturing a skin wound, setting up a drip or doing a lumbar puncture.

In short, we budding doctors lead a charming existance indeed. Our only consolation is that this is the time we can enjoy ourselves (Ha!) because very soon we'll be responsible, hard-working doctors with not a minute's peace, and not a drahm of gratitude from a clientele who claim that what we cure is in fact due to St. Rita, and what their negligence unfortunately causes is due to us "butchers". Which brings us to the question I sometimes keep asking myself. "What on earth made me choose Medicine as a career?"

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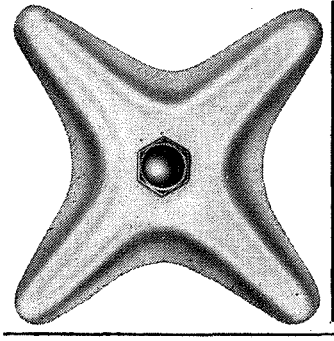

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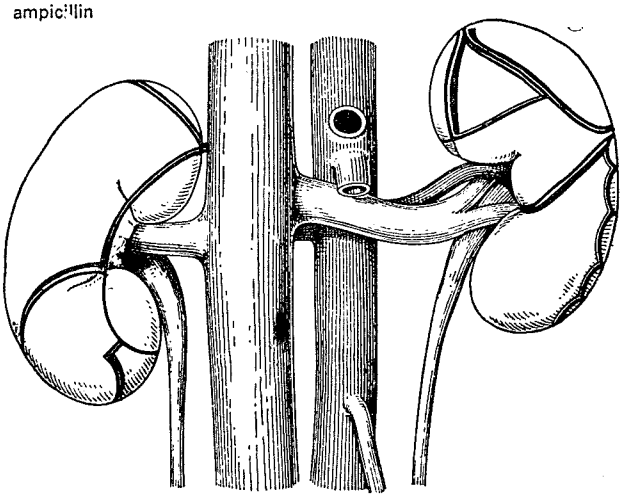

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THE HYPOTHALAMO HYPOPHYSEAL SYSTEM

By CHARLES A. GAUCI

The hypothalamo hypophyseal neurosecretory system is concerned with the production and secretion into the blood stream of the two hormones—oxytocin and vasopressin: the former is an uterine muscle contractant and lactogenic principle, the latter having important antidiuretic effects.

FUNCTION OF THE HORMONES

The effects of these two hormones show a considerable amount of overlap: both seem to exert the following functions:

a)	uterine muscle contraction	Principally oxytocin
b)	milk ejection	
c)	anti diuresis	Principally vasopressin
d)	vasoconstriction	

Vasoconstriction is only brought about when vasopressin is administered in pharmacological doses: in the body it is not secreted in sufficiently large amounts to cause vasoconstriction. Thus the name vasopressin usually ascribed to it is a misnomer. The principle effect of this hormone under physiological condition is antidiuresis (water conservation), so that a better name for it is Antidiuretic Hormone (ADH), and it is as ADH that we shall consider it in this article.

SYNTHESIS OF THE HORMONES

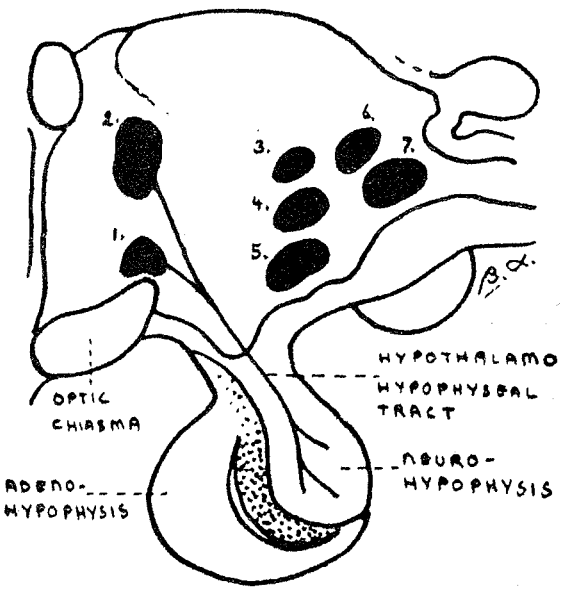
Sharrer and Sharrer attributed the synthesis of these two hormones to the supraoptic and paraventricular nuclei of the hypothalamus(1). They observed that the soma of the neurones in these nuclei apart from having all the characteristics of nerve cells, have also the characteristics of glandular cells being vacuolated and granular. These granules were shown by Bargmann to stain with Chrome Alum Haematoxylin (Gomori's stain) abbreviated to CAH i.e. they are CAH-positive (2).

There exists a definite axonal tract (the hypothalamo hypophysal tract) between the supraoptic and paraventricular nuclei of the hypothalamus and the neurohypophyses, and this CAH-positive material has been shown to move down the tract to the neurohypophyses(3).

NEUROSECRETION

The foregoing considerations raise the possibility of neurosecretion, and this has been the subject of a number of interesting experiments.

DIAGRAMATIC SAGITTAL SECTION THROUGH THIRD VENTRICLE TO ILLUSTRATE POSITIONS OF NUCLEI.



1. SUPRA-OPTIC NUCLEUS
2. PARAVENTRICULAR NUCLEUS
3. DORSIMEDIAL NUCLEUS
4. TUBER NUCLEUS
5. VENTRIMEDIAL NUCLEUS
6. LATERAL NUCLEUS
7. POSTERIOR NUCLEUS

1. Hild and his co-workers(4) cut through the hypothalamo hypophyseal tract and found, that whilst the part of the tract above the lesion showed an accumulation of the CAH-positive material, the part below it was devoid of such material.

2. Hild(5) obtained extracts of ADH and oxytocin from various sites of accumulation of CAH-positive material i.e. from the soma, axons and neurohypophysis itself.

3. Leveque and Sharrer(6) found that the amount of CAH-positive material down the tract bore a definite relation to the osmotic state of the plasma, i.e. it was abundant when the body was in a state of dehydration, and virtually absent in the presence of a water load.

4. Arnott and Sloper injected 35-S Cysteine into the cisternal space of an animal and subjected the dead animal to autoradiography. They found that the radio active material was rapidly taken up by the supraoptic and paraven-

tricular nuclei, and only after some time did it appear in the neurohypophysis. This cannot be due to a lack of blood supply of the neurohypophysis as this is well outside the blood brain barrier. Hence the cysteine must be actively used up by the supraoptic and paraventricular nuclei; in fact, cysteine is incorporated into the hormonal percursor, and is then converted to cystine.

From the above experimental data, one can readily appreciate the intimate relationship of the CAH-positive material with the octapeptide hormones, the former was demonstrated however, to be chemically distinct from the latter by various workers(7). The CAH-positive material is thought to be the carrier of the hormone precursor(8), which is synthesized in the soma of the neurones of the supraoptic and paraventricular nuclei, and which is carried down via the hypothalamo-hypophyseal tract to the neurohypophysis.

The endings of the tract are in intimate relationship with both the pituicytes (modified astroglia, abundant in the neurohypophysis) and the blood vessels of the neurohypophysis(9). It is thought that the hormone precursor passes to the pituicytes, which break it down into its active octapeptide form, and send it into the blood stream when the need arises(10). The neurohypophysis is an excellent storage depot for the hormone, as it is well outside the blood brain barrier(11), and has a very rich blood supply(12), as well as very permeable capillaries, so that the active hormone(s) may swiftly be ejected into the blood when required.

Evidence is now accumulating, suggesting that the hormone precursor is not only synthesized in the soma of the supraoptic and paraventricular nuclei, but also in their axons i.e. apart from axoplasmic streaming, there is a progressive synthesis of the hormone precursor(13).

CONTROL OF THE SECRETION OF ADH AND OXYTOCIN

It was shown by Verney in his "Carotid Loop" experiment, that the osmotic pressure of the plasma solutes is the factor which controls the secretion of the posterior pituitary hormones. The osmotic pressure acts on what Verney called osmoreceptors, and these then relay the stimulus to the neurohypophysis.

By means of intradural ligation, Verney and Jewell, in 1953, demonstrated that the osmoreceptors are situated in some part of the prosencephalon(14). Verney noticed small vesicles situated in the supraoptic nucleus of the dog and ascribed to them the function of osmoreceptors(15). This assumption seems improbable.

The exact site of these osmoreceptors is as yet not known, though some very interesting work in this direction was carried out by Sawer. Abraham and Pickford (1954) showed that upon injection of hypertonic saline, the posterior pituitary secreted oxytocin and ADH in the ratio of 30:1. Thus, there occurred a rise in intramammary pressure. Why there should be a rise in intramammary pressure as a result of an osmotic stress, is a mystery. Now Sawer(16) cannulated the mammary duct of a rabbit, and inserted bipolar electrodes deeply into the ventral telencephalon between the olfactory tubercle and the lateral preoptic area. He noticed, upon administration of an osmotic stress, an increased rate of firing in the neurones here, immediately before any rise in intramammary pressure: transection of the midbrain did not affect the firing or milk ejection response. Thus he concluded, that the osmoreceptors are situated here. This evidence however, is not conclusive.

An interesting point is, that oxytocin is also secreted in the male, but its function is not known: a similar state of affairs in the male exists with the anterior pituitary hormone prolactin.

Olivecrona found that bilateral destruction of the paraventricular nuclei in the rat results in a loss of extractable oxytocic material from the neurohypophysis. A neurohypophysis completely lacking oxytocin shows a normal pressor content(17). Again, an application of an osmotic stress was seen to accelerate the rate of firing of the supraoptic units and inhibit those of the paraventricular units(18); the direct stimulation of the paraventricular nucleus carried out by Cross(19), resulted in a marked milk ejection response.

This evidence would suggest that the supraoptic nucleus is concerned with ADH production, while the paraventricular nucleus is concerned with oxytocin production. There cannot be any clear cut line of demarcation however. Thus, although in the face of osmotic stress, there occurs an increase in the spike frequency in the cells of the supraoptic nuclei, relative to the paraventricular nucleus, more oxytocin than ADH is in fact secreted (see above).

Many factors other than the osmotic pressure of the plasma solutes, stimulate the secretion of the posterior pituitary hormones: a decrease in the extracellular volume as occurs in haemorrhage, for example, will stimulate secretion of the hormones (acting on stretch receptors in the great vessels). But the principal stimulus for secretion of these two hormones, is the osmotic pressure of the plasma solutes.

It may be pointed out as a matter of historical interest, that various workers, principally Abel, postulated that oxytocin and ADH are in

fact a single hormone exhibiting both oxytocic and antidiuretic activities (the "Unitarian Theory)(20). The existance of two individual hormones was proved by a number of different workers, but principally by du Vigneaud(21).

SUMMARY

The probable sequence of events leading to the release of the two octapeptide hormones in the face of an osmotic stress may now be summarized into four basic stages.

1. The osmotic pressure of the plasma solutes acting via the blood in the internal carotid artery stimulates the osmoreceptors in the ventral telencephalon.

2. The osmoreceptors stimulate the supraoptic and paraventricular nuclei: these two nuclei are probably producing the hormone precursor all the time, storing it in the pituicytes.

3. The stimulus is relayed down the hypothalamo hypophyseal tract to the pituicytes.

4. The pituicytes then release the active octapeptide into the blood stream.

Upon reaching the kidney ADH increases the permeability of the distal convulted tubules and collecting duct, so that water may pass out of the nephron and enter the interstitium, from where it is conducted away by the blood vessels. Hence water economy is achieved.

INTERRELATION OF THE ANTERIOR AND POSTERIOR PITUITARY

The antidiuretic action of ADH is abolished by severence of the pituitary stalk, but an increased excretion of water occurs only as long as there is an intact ANTERIOR pituary. In man, lesions leading to the destruction and degeneration of the neurohypophysis, can result in Diabetes Insipidus ,provided that there is an intact anterior pituary. Diabetes Insipidus is characterized by a polyuria of up to 20 litres per day, the urine excreted being very dilute and having a very low specific gravity: this polyuria is coupled naturally with a severe polydypsia.

There exists a relationship between ADH, the anterior pituitary and the corticosteroids relative to diuresis, but it is not well understood(22). It has been shown that fibres from the supraoptic and paraventricular nuclei of the hypothalamus containing CAH positive material, pass to the pars tuberalis and possibly also to the pars distalis of the anterior pituitary. These fibres come into contact with the radicles of the hypophyseal portal vessels in the median eminence and infundibular stem. The corticosteroids appear to have a direct effect on the action of the neurohypophysis, and it is possible that they are involved in the activation of the neurohypophyseal hormones. There also appears to be some connection between ADH and ACTH formation(23).

CONCLUSION

The mechanism of the hypothalamo-hypophyseal system is far from clear; there is a great deal of overlap with other humoral and neural mechanisms. At the moment the neurosecretory origin of the posterior lobe hormones remains the most attractive hypothesis. It offers a clear and reasonable explanation for the lack of secretory elements in the neurohypophysis and for the antidiuretic-pressor substance in the hypothalamus as well as for the very existence of the hypothalamo-hypophyseal tract itself.

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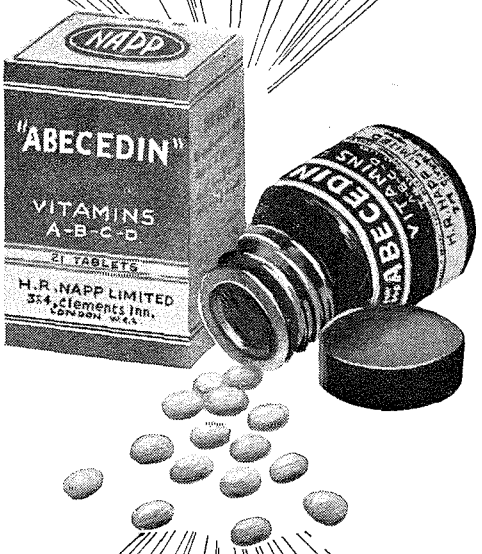

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STUDIES OF THE INTRACELLULAR LOCALISATION OF ENZYMES

CATHERINE SALISBURY, B.Sc.
Department of Physiology and Biochemistry
Royal University of Malta

In the first studies of tissue metabolism, metabolic reactions in cells and tissues were determined and then quantitative measurements of enzymes evolved. More recently the distribution of enzymes within the cell has been studied. This has been made possible by the production of more sophisticated apparatus. The knowledge of the localisation of enzymes within the cell in relation to its stucture is very important for the understanding of the functioning of the cell.

In 1839 the cell was identified as the fundamental unit and in this year Schleiden and Schwann independently propounded their cell theory. Now it has been shown that the cell is not just a limiting membrane enclosing a blob of cytoplasm, but instead contains a number of highly organised structures which vary between cell type. Although there is no such thing as a typical cell, some order can be found amongst the complexity. Studies of the structure of the cell have been greatly advanced due to the electron microscope, which has a very high resolving power. The instrument's main disadvantage is that electrons can only cross an extremely high vacuum, and it is therefore impossible to examine living cells. They must be killed and completely dehydrated before use, which gives a risk of distortion of the structures.

When it was known that the cell contained smaller structures, scientists wished to know where a particular enzyme was located within the cell. This has been studied by two main methods: (1) microhistochemistry and (2) biochemistry.

Histochemical methods may be regarded as developing from the staining techniques of classical histology since they depend on the liberation of a staining substance in tissue sections as a result of enzyme action, followed by microscopic examination of the stained tissue. In earlier studies the tissue sections were dehydrated by treatment with acetone and then embedded in warm paraffin wax. This denatures most enzymes, so recently tissues are quickly frozen and sectioned at low temperatures. There are certain limitations on the histochemical approach which are as follows:

(1) The product of the enzyme reaction must stay at the site of the reaction, so a fixing substance may be necessary.

(2) A staining compound is necessary, but must not interfere with the reaction.

(3) The fixing and staining compounds must be able to enter the cell freely without disrupting the integrity of its structure.

Because of these limitations the method is only useful for a few enzymes. An example of such an enzyme is acid phosphatase. A lead trapping agent is used which combines with the phosphate ions liberated in the reaction to give lead phosphate, an electron dense compound which will show up under the electron microscope.

The biochemical approach consists of isolating the enzyme and then studying the reaction it catalyses. The first stage is to obtain a cell-free homogenate where the cell membrane has been disrupted but the membranes around the intracellular particles remain intact. The procedure for this varies for different cell types, so a standard method has been developed for each. There are several ways of disrupting the cell membrane including (1) stress by velocity gradients, (2) alternate freezing and thawing, (3) enzyme action, and (4) sonication. Method (1) can be standardised most easily and is the preferred method. Methods (2) and (3) are harder to standardise and more random in their action.

The best method involving stress by velocity gradients is by use of a rotating pestle and mortar. An example of this is the Potter-Elvehjem homogenizer in which the mortar is moved up and down by the operator, past a rotating cylindrical pestle. The maximum rate of shear is at the point of contact with the liquid and rotating pestle, which will distort the cell membrane and so disrupt it. The disadvantage of this homogenizer is overheating which could destroy an enzyme by denaturation of its protein structure. The Potter-Elvehjem homogenizer is however most commonly used. The procedure can be standardised by the number of strokes and the speed of the pestle's rotation.

The medium used for the homogenate has a profound effect on the cytological and biochemical properties of the particulate components. Those of the mitochondria in different media have been studied most thoroughly and have therefore served as a basis for the selection of media.

It is very important that during homogenization the intracellular membranes should remain intact. There are several methods for checking this. One should get reproducible results for the distribution of enzymes within the cell. When an enzyme is located within a cellular particle, its activity will be latent, i.e. it will only appear after the membrane around this particle has been disrupted. Therefore if the homogenate is a good one, these enzymes

should display latency. Fractions of the cell can be obtained by differential centrifugation. The distribution of several enzymes in these fractions is known, so that if the homogenate is good, the distribution pattern will be identical to the standard. If however the membranes have been disrupted within the cell, this distribution will alter, the activity probably being found mainly in the cell supernatant.

Obtaining these cell fractions, which I have mentioned above, is the next step in the procedure for the localisation of enzyme activity. The various structural components of a homogenate sediment in the centrifuge at different rates, primarily due to their difference in size, and can be separated by a series of spins usually at increasing speeds. By resuspending the centrifuged pellets and centrifuging again, a number of fairly homogenous fractions can be obtained. This method is termed differential centrifugation.

As a result of the work by Schneider, Claude, Hogeboom, Dounce and others a more or less standard fractionation scheme has been adopted to give the following fractions: (1) intact cells and tissue debris, (2) nuclei, (3) mitochondria, (4) lysosomes, (5) microsomal fraction, (6) supernatant.

Many enzymes can be studied by this method, several on the same preparations thus making comparisons of activity and requirements easier. The fractionation scheme must vary with different tissues because of the different cell structures, and now schemes have been devised for nearly all tissues from rat liver cells to bone.

There are however several disadvantages in this method. Separation from its natural environment may affect the enzyme. Fractions may be contaminated by particles from another fraction. In addition the enzymes in a fraction are not necessarily all present in the same kind of particle. For example, De Duve found that the mitochondrial fraction contained lysosomes. He was studying the distribution of alkaline phosphatase activity and found that the total activity of the cell fractions was less than that of the homogenate. On leaving in a refrigerator overnight, the activity increased in the mitochondrial fraction so that the total activity equalled that of the homogenate. Careful differential centrifugation separated these particles, the lysosomes being smaller than the mitochondria sedimented more slowly. It was much later that cytologists were able to identify these structures under the electron microscope. Mitochondria seem to be homogenous with respect to size and enzymic composition, but lysosomes although enzymatically homogenous have a wide range of size, and this makes it difficult to obtain them free from both mitochondria and microsomes and *vice versa*.

Another example where more than one structure may be present within a fraction is in the microsomal fraction, which contains fragments of endoplasmic reticulum either rough or smooth. The rough membrane has ribosomes on its surface which are the site of incorporation of amino acids to form proteins. This fraction also contains vesicular elements coming from the Golgi, plasma, and nuclear membranes.

To conclude the disadvantages of the homogenate technique, one may mention that when there are many cell types present in the homogenate, the enzymic activity cannot be associated with a particular cell type as in the histochemical method, where the cell can be identified under the microscope.

To obtain a complete picture of the localisation of enzymic activity, each fraction is analysed by sedimenting in a density gradient which includes the value of particle density associated with the enzyme. After equilibrium is reached particles rest in a band centered round their own buoyant density. Each layer is then analysed for enzymic activity.

The usual swing-out heads used in centrifuges introduce errors by disturbing the density gradient on swinging up to the horizontal when gathering speed, and down again to the vertical at the end of the spin. This is eliminated by a new type of centrifuge using the method of zonal centrifugation, whereby the density gradient and fraction under test can be introduced and removed whilst the centrifuge head is still spinning horizontally.

Interpretation of results requires more care. Morphological and biochemical properties are assigned to the fractions bearing in mind the components of the original homogenate and the fractionation techniques orginally employed. These components of a homogenate may finally be equated with certain intracellular entities.

It is important to show that the sum activity of all fractions equals the activity of the unfractionated homogenate. The possibility that inhibitors or activators may be present must be considered. In such a case the distribution of enzyme and of inhibitor or activator may be entirely different, with the result that the sum of activities of the fractions will be greater or less than the activity of the homogenate. It is then necessary to measure the activities of the fractions in all permutations and combinations, as well as separately, in order to determine the localisation of the inhibitor or activator as well as to eliminate the possibility of denaturation during the isolation procedure. When these

requirements have been met, the localisation of an enzyme in a cellular component is indicated in three ways: (1) a large percentage of the total activity of the homogenate is in that fraction; (2) the specific activity of the fraction is several times greater than that of the homogenate; (3) the specific activity of the fraction remains constant upon repeated sedimentation. These criteria have been satisfied for only a few enzymes.

The identification of the subcellular fractions can be performed either by examining under an electron microscope, which if the fraction has been carefully prepared should show only one type of structure, or by enzyme assay with an indicator enzyme whose distribution is well known.

De Duve has pointed out the two fundamental assumptions in biochemical studies of the intracellular distribution of enzymes. They are that an enzyme is localised in one intracellular site, and that populations of subcellular fractions are enzymatically homogenous. Some untenable assumptions may be mentioned, for instance, that analogous particles in different cells, tissues or organisms should have the same size, shape or destiny and would therefore be found in the same fraction. Because this cannot be assumed, the fractionation scheme employed depends on the tissue concerned. In like manner it cannot be assumed that the intracellular localisation and certain properties of a particular enzyme such as latency remain invariant from cell to cell. This would have to be established for each application.

The absence of a certain activity from a certain fraction does not necessarily mean that the intracellular component does not possess the enzyme because the activity may have been detached or liberated during fractionation. Many nuclear and some mitochondrial and lysosomal activites are of this kind. Conversely, the presence of an activity in a fraction does not guarantee its original association with a particle *in situ*. The enzyme may have originally been soluble or detached from one particle type, only to become attached to a second particle type. For example, the attachment of basic proteins such as cytochrome c to ribonucleic acid of ribosomes is known to occur.

One of the most striking results from studies of the intracellular localisation of enzymes is that in many important metabolic systems all the enzymes and coenzymes are present in the same particle. For example, all the components of the oxidative chain are found in intramitochondrial particles. This however has been overstressed in the past, for only two of the enzymes of the tricarboxylic acid cycle are found entirely in the mitochondria. The rest are mainly localised in other fractions.

As I have shown, there are many pitfalls in the interpretation of results, but with care the biochemical method is very useful in conjunction with cytology for advancing our knowledge of the intracellular distribution of enzymes.

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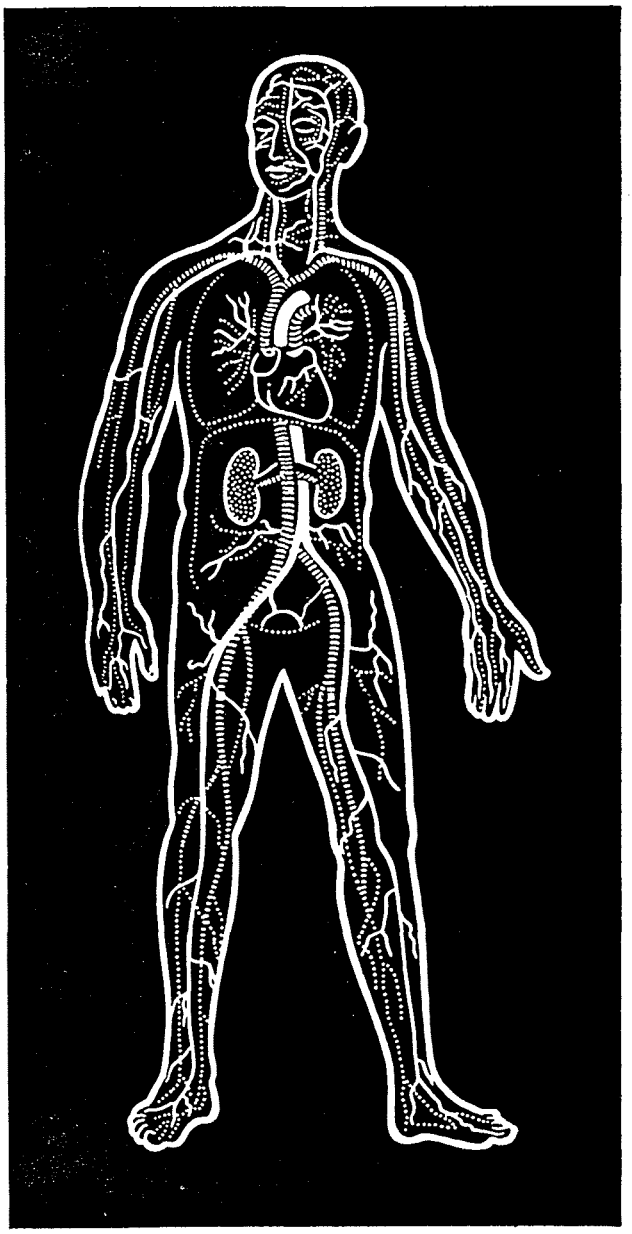

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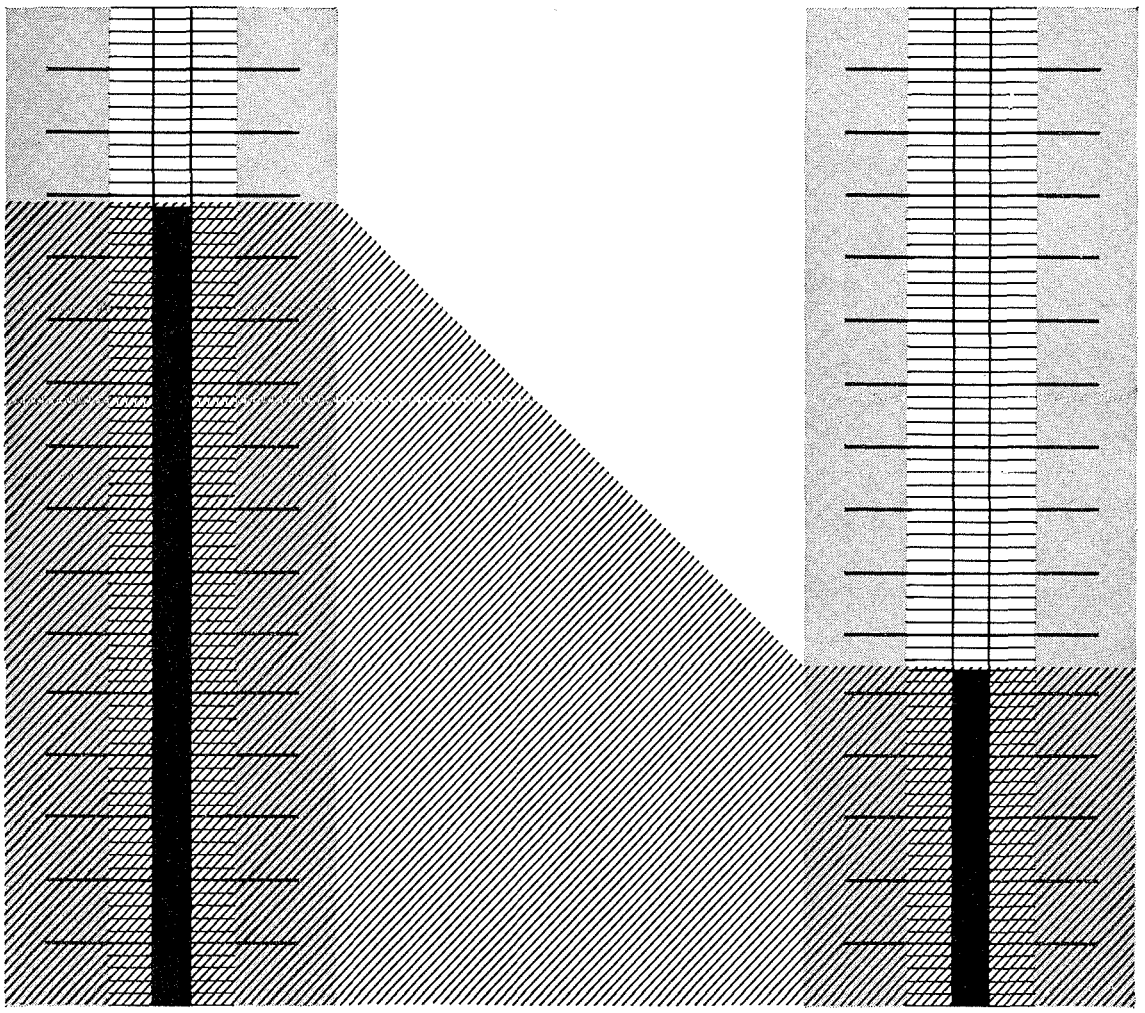


MISCELLANEOUS

Since the last edition of our journal, an important change has taken place in the department of Medicine, Professor W. Ganado retired. Those of us in the Final Course only knew him for a short time, but it was long enough for us to appreciate the accompanying cartoon of him drawn by Timothy Rudman, from the Royal Free Hospital, who was clerking with him in Summer 1966. We are sure that this event will not curtail his active life.

With equally regrettable delay we would like to welcome Professor J. V. Zammit Maempel as the new head of the department of Medicine. We thus continue the association we started when he was Professor of Pharmacology, Materia Medica and Therapeutics.

APPEAL

If any of our readers has a complete set of past issues of the "Chest — Piece", we would be very grateful if he would contact the editor, as we are trying to build up a collection for the Medical Library.

SUMMER SCHOOL

Literature and application forms have been received for the Medical Summer School in Scandinavia, and it is hoped that students will take this opportunity to go and visit the Scandinavian countries and benefit from their systems. Reference to the 1967 issue of our magazine will prove that it is an invaluable experience. Particulars can be obtained from the Exchange Officer.

NEWS FROM THE PHARMACEUTICAL INDUSTRY

A new Medical Research Centre of Boehringer Mannheim was inaugurated last December. This Centre is the first of its kind, keeping all kinds of experimental animals under the same roof as the laboratories. The animal building has air conditioning throughout and an artificial climate and day/night rhythm are provided. The meticulous care that has gone into the planning of this Centre should ensure the reliability of the experimental results.

MEDICAL EDUCATION

This item is more in the character of a "Stop Press" as events have taken place just before going to print. On the 5th February the Clinical Course met informally to discuss the "Minor subject examinations" and it was agreed, by a certain majority, that these should be held in March and rejecting the idea that they should be incorporated with the major subjects. If the arguments that were put forward are followed logically it seems that the majority would welcome Orthopaedics, Anaesthesia, E.N.T. and Paediatrics as separate examinations. Let us hope that in the near future our Medical School will be blessed with a more imaginative course who will think Medicine in terms other than memorising from books.

FOOTBALL

Allow me to introduce you to the football team who call themselves 'Gray's Bone-crushers'. They are a group of young IV year medical students with lots of initiative and stamina which have proved by the fact that in spite of all the difficulties encountered e.g. studies, girls, bad weather etc., they did their utmost to surmount them and manifest the talent of our beloved faculty.

The team usually lines up as follows:

Alfred Magri Demajo; Joe Carabott Damato,

Mark Vella Bardon; Klaus Vella Bardon, Valhmor Zammit, Raymond Zammit; Tony Debono, Tony Leone Ganado, John Sammut Tagliaferro, John Aquilina, Emmanuel Fiorentino.

Substitute: Anton Bencini.

Since its foundation the team has not suffered a single defeat. Some of the results attained in various matches were:

vs	Dante Alighieri	5-3		
vs	1st Year Science	5-2		
vs	Architecture	3-3	0-0	
vs	Medicine VI Year	3-1	2-0	3-3
vs	Architecture-Arts Combined	3-1		

The team adopts a 4-3-3 system basing its play on a sound defence, and efficient and brainy (??!!!) midfield, and a good getting (flukes included) forward line. No time is wasted by the players, not even during matches because during periods of respite (when we get them) out come our pocket-anatomy books and we all gather round with our skulls and other various bones and discuss any latest topics. With such a team, and supplemented by players such as Antoine Schranz, Joe Azzopardi, Leo Said and others (VI Year Medical Students) we hope to carry off the much coveted inter-faculty championship cup. This is easier said than done because stiff opposition offered by the Arts team together with the hoodoo which has been dragging us for a long time seem to be two of the most difficult obstacles yet to be overcome.

However, I am confident we will do it this time. If not.....! I wouldn't dare forecast the drastic consequences.

EXTRACHROSOMAL FACTOR DETERMINANTS

B. I. ANAZODO B.Sc. (St. And.), Dip. Micro.

In the early days of bacteriology even eminent bacteriologists like Koch, Cohn and Migula believed in the doctrine of monomorphism—the constancy of bacterial species—despite conflicting observations made by Pasteur on attenuated strains of Bacillus anthracis. Any variants that were discovered were dismissed as contaminants or as "Aberrant" or "Degenerate" or "Involutionary" forms. Nowadays they might have called them "L" forms. The concept of variation began to be taken more seriously after the discovery of lactose-fermenting strains of E. coli which appeared in the papilla on the surface of a non-lactose fermenting strain, because such variants could hardly be contaminants or aberrant. There is evidence about another type of variation brought about by small genetic carrying particles in the cytoplasm. This has been shown by their non-random appearance during the growth of culture. These genetic-carrying particles which lie extrachromosomally are known as plamids and episomes.

These are accessory structures and represent addition to the total genetic content of the cell. Despite their size, they play a great part in bringing about variation which is a great nuisance in the action of drugs. This new class of factor-determining bodies was discovered in the past decade so that our knowledge about their action is merely superficial. The word episome was introduced in 1958 by Jacob and Wollman and means "on the body". They can exist in two alternate but often mutual states:

i) the "integrated" state—the element exists on some point on the chromosome and multiplies synchronously with the chromosome;

ii) the "autonomous" state—the episome exists in the cytoplasm and multiplies independently from the chromosome and frequently faster.

GENERAL PROPERTIES OF EPISOMES AND PLASMIDS

i) Under normal conditions the properties they control are non essential.

ii) They can be present in either the integrated or autonomous state—generally the integrated state precedes the autonomous state.

iii) Plasmids exist autonomously but not in the integrated state.

iv) During conjugation, episomes can be transmitted independently of the bacterial chrosomes, either by mere transfer of those lying in the cytoplasm autonomously or elution of those already integrated.

v) Episomes can be eliminated spontaneously. They can also be eliminated by treatment with certain reagents like acridine dyes, divalent salts of Cobalt and Nickel, and certain oxidising agents, particularly periodates. The elimination occurs mainly only when they are autonomous and not when they are integrated.

There are four well established units considered as episomes and several which are still being contemplated.

The forms established are:

i) Genetic material of temperate bacteriophages.

ii) Sex or fertility factors.

iii) Colicinogenic factors.

iv) Factors responsible for the infectious heredity of multiple drug resistance.

Other factors thought to be brought about by episomes are:

i) Factors concerned with lactose fermentation and utilisation.

ii) Fimbriation or piliation factor.

iii) Ability to form spores in Bacillus and probably Clostridia-Sporogenic factors.

iv) Penicillin resistance in Staphylococcus and probably other genera.

A *Temperate Bacteriophage* is one which once in the bacterial cytoplasm, its (phage) nucleic acid provides the genetic information for the synthesis of a series of specific enzymes. which in conjunction with the pre-existing metabolic machinery of the infected cell, catalyze the formation of more phage nucleic acid and phage protein. Only in the late stages of the infectious process are these structural subunits assembled within the infected cell into new virions. The instruction by some phages destroy the nucleus whilst instruction from others do not destroy the nucleus, viz: dependent ones. Immature phage do not appear until there is a pool of precursor materials which are formed in a given order. Amongst the pool are enzymes and phage lysozyme. Although temperate bacteriophage may give the production of active phage and its release by lysis as in lytic cycle, generally it persists in an inactive form indefinitely and it becomes hereditary in the bacterial host cell. A strain of bacteria infected in such a way that active phage is not

produced but persists in an inactive form is said to be lysogenic and the phenomenon is known as lysogeny.

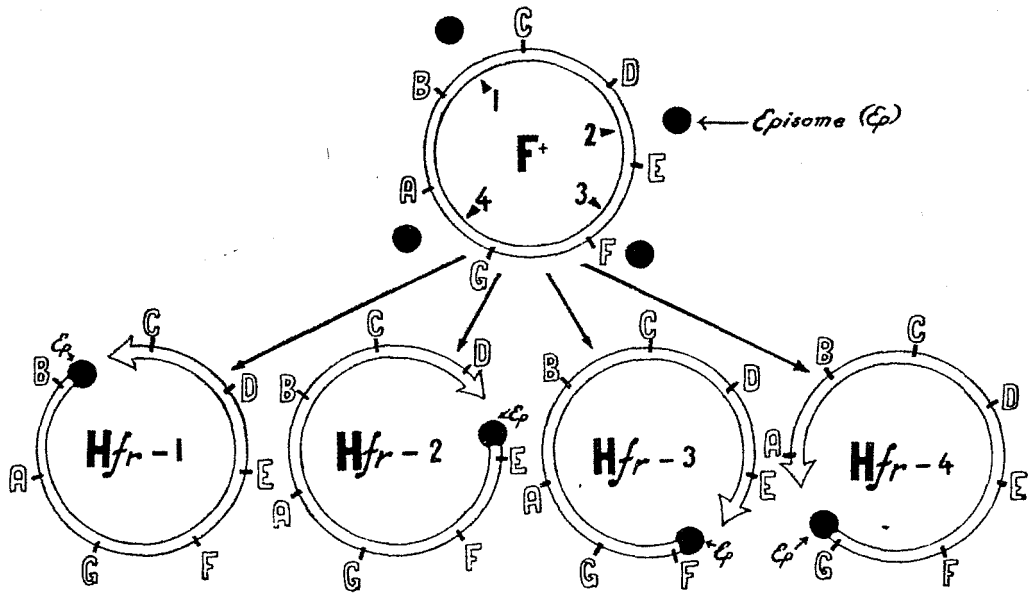
In the lysogenic state the virus is integrated and in the integrated state the genetic material of the phage becomes a constituent of the bacterial cell and behaves as such. Also in this state, the normal viral function is not expressed; the phage persists within the bacterial cells and is transmitted from mother to daughter cell during division and so is not affected by serial culture in antibody containing medium. In bacteria infected by temperate phage—lysogenic bacteria—the entire directive mechanism of the host cell is supplemented by that of the virus unlike the infection of bacteria with virulent phage where the entire directive mechanism appears to be replaced by the virus. In consequence, characteristics of the bacterium, ordinarily regarded as inherent, biologically fundamental properties of the cell, may be attributable to or modified by the presence of phage in the lysogenic state, e.g. the diphtheria bacillus (Corynebacterium diphtheria) is distinguished from its closely similar diphtheroid (Corynebacterium hofmanii) by its production of diphtheria toxin. Toxigenicity, however, has proved to be a characteristic only of lysogenic diphtheria bacilli and may be removed by the abolition of the lysogenic state or conferred on diphtheroid bacilli by making them lysogenic. It appears to be definitely established that all toxigenic diphtheria bacilli are lysogenic, though all lysogenic strains are not necessarily toxigenic and that the induction of toxicity is inseparable from phage. Furthermore, characteristics of a lysogenic bacterium, either seemingly pre-existing, such as biochemical characteristics or content of specific antigens, or acquired, such as drug resistance, may be transferred to another bacterium making it lysogenic with phage derived from the first bacterium.

Virulent phage may act as a transducing agent under appropriate conditions (transduction is the phenomenon by which genetic material is conveyed to the recipient by phage grown on the donor bacteria). The recipient cells (bacterial) acquired a new character and are the transductants. Transductants may be complete or abortive. Complete transductants are recipients in which the donated genetic component is integrated in the recipients genome, e.g. when phage grown on a prototrophic donor strain is allowed to infect a cysteine—requiring recipients—prototrophic transductants are obtained which remain stably prototrophic on subculture. In abortive transductants, on the other hand, the donated fragments are not integrated and when the recipient cell divides, the fragments pass to only one of the two daughter cells and continue to do so on further divisions of each cell containing the fragments—the process of "unlinear transmission". Virulent phage may act as a transducing agent under appropriate conditions, but most often the transduction is by temperate phage.

SEX OR FERTILITY FACTOR

It has been discovered that there are two mating types in E. coli and that during conjugation one partner acts only as a genetic donor or male and the other as the genetic recipient or female. A male retains its fertility even after dying of streptomycin whilst the fertility of the female is destroyed by lethal agents. Thus, the male need not remain viable since its only function is to transfer D.N.A. whilst the female contributes genetic material and a complete viable cell. Both parental strains were equally sensitive to the drug as judged by survival. From this it was deduced that there was a one way transfer of genetic material from a donor (male) to a recipient (female) strain; the donor could be dispensed with once its function had been fulfilled, but the survival of the recipient in which the whole process of recombination and segregation took place was essential. Moreover, the fact that the recombinants inherited most of their characters from the recipient parent further suggested that the genetic contribution of the donor bacteria to the zygotes is fractional. It was then discovered that the donor state is genetically determined not by a chromosomal gene or genes as one might expect but by an infectious agent called the sex factor or "F" (fertility) agent which exists separately in the cytoplasm. This factor promotes conjugation between donor bacteria that harbour it, (termed F+) and recipient bacteria (F−) that lack it, followed by its own efficient transfer to the recipients which are thus themselves converted into F+ donors. When male F+ and female F− conjugate, many of the females are infected and converted to male. "F" agent is transferred only by cell to cell contact, never kills its host, and is never released into the medium as a virulent virus. "F" agent is thus autonomous. In a cross of F+ × F− the sex factor is transferred with up to 100% efficiency. All the progeny are F+. It seems that sex is highly infectious. Actual transfer of the chromosomal marker by F+ is a very rare thing—of the order of 10^{-4} (i.e. $1/10^{4}$). Mutant male strains which transfer their chromosomal genes with very high efficiency are known as "Hfr"—high frequency recombination. They are genetic donors, over 10% transferring chromosomal materials. When recombinants are isolated from

an Hfr × F− cross they are usually found to be F−. (Compare with F+ × F− cross). In other words, Hfr do not transmit infectious F particles but chromosome transfer has increased at least 1000 times and the sex factor is no longer present as an infectious agent. Most of the genetic traits are those of the recipient because it is usually found that most of the genes have been derived from the recipient (F−) parent and only a few characters from the donor parent. This is because the donor cell transfers only a part of its genetic complement during conjugation. The F+ to Hfr mutations are accompanied by the breakage of the chromosome which is often circular; one end of the broken chromosome becoming the leading point in chromosome transfer. There are many types of Hfr and in each type, the break has occurred at a different point so that each transfers its genetic markers in a different order. The order in which the genes are transferred is precisely the order in which the genes are arranged along the chromosome. What matters is where the break occurs.



HYPOTHETICAL FACTORS

A Lactose fermentation
B Methionine +
C Galactose fermentation
D Serine +
E Histidine +
F Proline +
G Coliphage
1, 2, 3, 4, sites of chromosome. One break in each chromosome.

The sex factor is attached to the chromosome at the broken end opposite the end which first penetrates the female F− during the conjugation. Thus if recombinants are selected which have received the very last marker to be transferred, e.g. Methionine in Hfr−1; Histidine in Hfr−2; Proline in Hfr−3 and Coliphage in Hfr−4; then the recombinants also receive sex factor and behave as Hfr males with the same order of gene transfer as their parent. The sex factor is thus transferred only as a chromosome marker being the last to penetrate the female cell. The sex factor is then borne in an integrated state.

METHOD OF STUDY OF SEXUALITY — INTERRUPTED SEX EXPT.

The transfer of genetic material between cells takes place across a bridge and the complete transfer takes about two hours. If the conjugating cells are agitated, as by treatment in a blender, before transfer is complete, partial transfer is found in the progeny. This allows for the determination of a sequence of hereditary units that suplement that deduced from evidence of linkage.

COLICINOGENIC FACTORS

Colicines are proteins or peptides in nature.

They are produced by enterobacteriaceae and are active against other members of the same family. Ability to produce colicine is governed by colicinogenic factor. Colicinogenic factors can be transferred from Col+ to Col− strain.

Evidence of the integrated state is scanty as it is always eliminated by treatment with such reagents like acridine dyes and divalent salts of Cobalt and Nickel. It is thus better described as plasmid rather than episome.

METHOD OF STUDY OF COLICINOGENIC FACTORS

Grow on nutrient agar about fifty colonies and assume that some are Col+. After 24 hours put this over a dish containing chloroform which serves to kill off the bacteria but not the ability to produce colicine. Cover the surface with thin layer of more agar and flood with an indicator which is sensitive to Col. Reincubate and if there is no colicine present there will be confluent growth, but if the original strain is producing colicine, it will diffuse out and produce a number of bare patches hence indentifying the original colonies as being colicinogenic.

SEXUAL HEREDITY OF MULTIPLE DRUG RESISTANCE

Recombination can be selected by means of antibacterial drugs, e.g. streptomycin, with drug resistance as a marker. Ledeberg and Tatum 1946, using E. coli strain K12 isolated prototrophic recombinants(1) of genotype a+ b+ c+ d+ from a mixture of reciprocally marked auxotrophic parent strains(2) a+ b+ c− d− × a− b− c+ d+ by plating on a defined medium lacking the growth factors (a), (b), (c), and (d). The use of doubly auxotrophic parental strains made it almost certain that the prototrophic colonies arose by recombination and not by back mutation because the probability of a double mutation in the same cell is the product of their individual probabilities. When these are 10^{-11} and 10^{-9} respectively ,the probability of both occurring simultaneously is 10^{-20}. Mutation to drug resistance is a serious cause of failure of chemotherapy, when only one drug is used but the risk of drug resistant strains emerging is generally reduced if two drugs are given at the same time, since doubly resistant strains are extremely rare. Occasionally, prototrophic organisms undergo mutation to auxotraphic forms; these can grow on simple medium only if this is supplemented with a particular nutrient compound, e.g. a particular amino-acid or vitamin. The specific nutritional requirements show that the organism has not the power of forming either the enzyme immediately responsible for the synthesis of the required compound or else an enzyme responsible for synthesis of same. Studies in mutations of this kind by Beadle and Tatum have indicated that the formation of each specific enzyme is determined by the action of a different specific gene; this is the "one-enzyme one-gene" theory.

Moreover, since recombinants never arose unless intact bacteria of both parental types were present, it was correctly assumed that the genetic transfer is mediated by cell to cell contact, that is by conjugation.

Sexual heredity of multiple drug resistance is also applicable to the enterobacteriaceae, especially to the pathogenic organisms fed on antibiotics like tetracycline, chloramphenicol, etc. Each factor is a resistance (R) factor and the transfer is controlled by Resistance Transfer Factor (RTF). They exist autonomously and in the integrated state and can be got rid of either physically or by chemicals. It was first discovered in Japan in 1957 in Shigella, and by using volunteers it can be shown that multiple resistant organisms occur in the gut. Resistance Transfer Factors are by definition episomes and like the sex factors of E. coli, to which some seem to be related both genetically and functionally, they mediate conjugation and can spread epidemically through populations of intestinal bacteria which lack them. Some of these factors can also rarely initiate chromosome transfer, but the equivalent of Hfr bacteria have not yet been isolated from strains carrying these factors. Resistance transfer factors are of considerable importance in medicine because they have picked up and incorporated into a single transmissible structure the genetic determinants of bacterial resistance to a wide range of antibiotics in common clinical use such as Kanamycin, Ampicillin, Streptomycin. As many as seven such determinants have been reported to be carried by a single transfer factor. These factors can become extensively disseminated among the normal flora of the intestine in human and animal populations, and thence be transferred by conjugation to a wide range of dangerous intestinal pathogens to initiate epidemics which cannot be treated effectively.

a) Other factors which are very likely to be determined by plasmids and episomes and which are occupying the energies of bacteriologists, biochemists and cell cytologists are—Fimbriation or Piliation factors—these factors allow

(1) Prototrophs: A strain with the nutritional requirements of the wild type strain.

(2) Auxotrophs. A mutant organism unable to synthesize a certain growth factor which can be synthesized by the wild type strain from the more elementary precursor sustances.

certain enterobacteriaceae which initially had no agglutinating ability to develop the power.

b) Ability to form spores in Bacillus and probably clostridium-sporogenic factor. This is transferred either by transduction mediated by phages or by conjugation.

c) Penicillin resistance: More fashionable and better studied is the mechanism by which cells acquire penicillinase activity from scratch by acceptance of extra-chromosomal factors (episomes or plasimds) which can carry the B-lactamase gene complex in a state which is partially autonomous from the chromosome, but they may otherwise function in the chromosomal genes.

In coliforms the penicillinase genetic system may constitute part of an extrachromosomal "R" or resistance factor, which, by linkage with "T" or transfer factor may pass from one cell to another spontaneously (not necessarily within the same species) (Datta and Kontomichalou, 1965).

In Staph aureus, "penicillinase" plasmids can be transferred from one cell to another—not spontaneously, but by transduction through an infecting bacteriophage (Richmond, 1965a: Novick, 1967). It is possible, with Staphylococci, for the cell to possess more than one type of penicillinase plasmid at the same time. In this organism it seems likely that the same or analogous penicillinase genes can, in certain strains, exist fully integrated on the chromosome, though there is yet no clear evidence that they can pass reversibly from the extrachromosomal to the chromosomal state like episomes.

At the present time, our knowledge of these bodies is meagre but it appears that no matter how infinitesimal this may be, it still is invaluable as these small bodies besides being widespread may be implicated in some disease like cancer and might yet be our guiding beacon in striving for the elucidation of the causation of so many diseases which to this very day lie beyond the reaches of the scientist's groping mind.

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MOGADON* AND NATURAL SLEEP

Striking advances have recently been made into the knowledge of sleep with the aid of the electroencephalogram, and it is now recognised that sleep does not consist of a quantitative reduction in cortical activity but of a qualitative change. Hypnotics, by their generalised depressant action on all the brain structures, greatly reduce cortical activity and sleep takes on an abnormal pattern. The development of Mogadon, however, has now made the treatment of insomnia possible without any significant lessening of cortical activity. The sleep induced closely resembles natural sleep; there is no disturbance of the normal rhythmical balance that exists between the sleep and wake processes and the patient will awake refreshed, without hangover and without mental confusion.

ROCHE

Mogadon the successor to the hypnotics

J 169 * registered trade mark for preparations containing 1,3-dihydro-7-nitro-5-phenyl-2H-1,4-benzodiazepin-2-one.

Agents: Cherubino, 64 Old Mint Street, Valletta

STUDENTS ABROAD — SUMMER 1967

MUNICH — (A. Sciberras and E. Morrison)

We were at the "Chirurgische Klinik der Universitat", a very large hospital for surgical patients. There we were attached to the Thoracic Unit and had a rather exacting schedule distributed over each day from 8.00 a.m. to 7.50 p.m. Monday to Saturday, the latter day being optional.

Each day we did routinely B.P. readings, taking blood samples, removing stitches, doing E.C.G.'s where indicated, and clerking new patients.

There were operations every day and the most frequent were: 1. Dilatation of stenosed mitral valves; 2. Blalock's operation for Fallot's Tetralogy; 3. Valve replacement by prosthesis (Starr-Edwards) in markedly incompetent aortic and mitral valves; 4. Implantation of subcutaneous pacemakers for correcting Heart-Block; 5. Pneumonectomy for lung cancer.

The operating theatres were crowded with specialised electrical equipment. Strategically placed TV screens displayed variegated "jigsaw puzzles", other bright-eyed gadgets blipped and beeped, a gamut of specialists all coordinating in complex manoeuvres, the steady hum of the heart-lung machine, all communicating to the surgeon the conditions of the patient now unrecognisable due to the camouflaging of wires, platings and sundry excrescences upon his body. Indeed it was an eye-popping experience for many students attached to this unit.

Relief from our world of mechanisation was provided by a well organised programme arranged by the student's association. Each summer over 200 students do clerkships in Munich. The cosmopolitan nature of this gathering can be evidenced from the small group attached to our unit—Czechoslovaks, Peruvian, Canadian, Finnish, Mexican, Polish, Greek, French, Maltese and Jamaican—and despite the devolutionary return to a hand-foot-face signalling, we did much to confirm man's gregarious nature. Thus included in the week-end programme, were mountain climbing, trips to Salzburg and Berlin, rafting on the Isar river, visits to museums, churches, University extra-mural centres etc., etc. and all at favourable conditions to the pocket.

Munich was undoubtedly enjoyable and despite our meagre armamentarium of German, we did manage on our more generously supplied English and the afore mentioned Esperanto.

COPENHAGEN (E. Morrison)

In view of the fact that this is the birthplace of IFMSA, there was not undue disappointment on noticing the relative negligence of the medical student's association in arranging some form of programme for the many clerking students.

A month of Pathology here at the Pathological Institute of Bispebjerg Hospital was rather informative. There was a daily schedule of P.M.'s in the morning and Histology in the afternoons, Monday to Saturday from 8 a.m. to 6 p.m. The average day had six P.M.'s and after the first day of observation I was responsible for two cadavers.

Through wind and rain in fog and bitter cold, each day I made my way to the P.M. room to do my share of carving. Scrupulously following the methods of that house, one worked at the viscera that had previously been removed by the attendants.

Binocular microscopes opened a new world for my Histology and in addition to P.M. sections, biopsy specimens were examined.

The weather aside, a clerkship in Copenhagen is absolutely breathtaking. There are numerous places of interest to see, but prices readily surmount the resources of the pocket. Tram and bus fares, admission fees, cost of souvenirs, all outrageously expensive from the student's point of view. This is where student association activity is lacking.

PAVIA (Rose Galea)

Medical clerkships are held at the Policlinico San Matteo, comprising a number of clinics, extremely clean and laid out in spacious and well-tended grounds.

I spent three weeks in September at the clinic of "Medicina Semeiotica", a building having 60 beds. Together with three local students, I did a daily ward-round in the mornings and was expected to take B.P.'s, pulses, report on the progress of the case and take plood samples. Senior students would take histories of new cases and set up drips. After the ward-round our time was spent in the laboratories carrying out blood counts and urine analysis.

My free time was spent answering questions about Malta and our way of life. I found that most of them had wrong ideas about our politics, and education, though in the E.N.T. clinic they had a Maltese, Prof. Mario Cherubino, who proved to be most helpful. He is doing research on melanocytes in the ear and would like to take this opportunity to convey his regards to the many friends he made during the Congress of Catholic Doctors in Malta.

Pavia boasts of the oldest university in the world and the town is rich in old buildings. It is in the Lombardy plain and only half an hour's train journey from Milano, 2-3 hours from Genova and the lakes (Lago di Como).

September is not a good month to go as

most of the local students are busy with examinations. The accommodation left much to be desired as the "Pensione" where I was lodged lacked hot water and heating and was generally quite unsatisfactory. Meals were had at a restaurant with which the students had an arrangement, and the food was excellent and sufficient.

PALERMO (J. M. Bozzino)

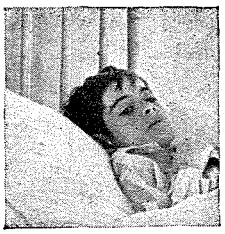
Arrangements for me to go to Palermo were made at the last minute and that I managed to go does great credit to the Exchange departments concerned.

My aim was to attend P.M.'s but there were none performed during my stay there, as things are very quiet in Sicily during August.

I was staying at the "Instituto di Medicina del Lavoro", which formed part of the "Policlinico". This was a very busy and somewhat overcrowded clinic, caring mainly for workers and their families, thus the age-group of patients was lower than in the medical wards of our hospital. I was sleeping in the same room as the doctor on duty, so some nights could be quite hectic. There were no specific duties alloted to me, but on the other hand there was nothing I was told specifically not to do. The doctors were all studying for some specialist examination in Medicine and seemed to be rather overworked by their superiors. One of the things that did strike me as an example of Italian ingenuity, was their use of a decompression chamber for hyperbaric oxygen therapy.

Sicily is a very beautiful country, especially the area round Palermo which has magnificent mountains. The cities show the influence of eight civilizations, and the monument that overshadows all others is the Norman-Byzantine Cathedral of Montreale.

Anxiety is in every ward

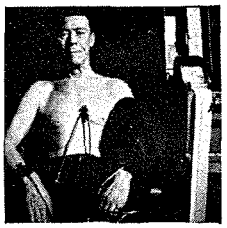


and every clinic

preceding birth

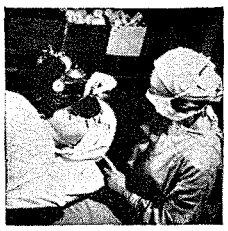
anticipating

death



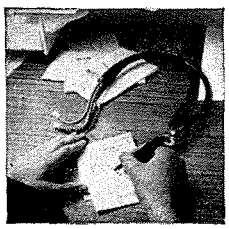
complicating treatment.

But where there is anxiety



in hospital or

general practice



there is Librium*

ROCHE

*Registered trade mark for preparations containing chlordiazepoxide

J 203

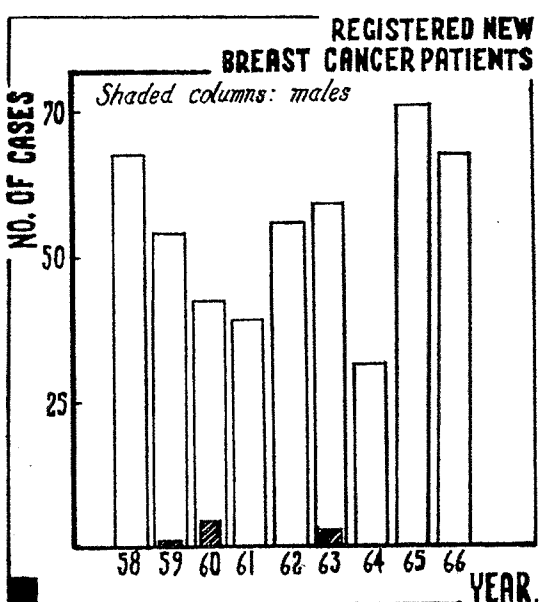
Agents: Cherubino, 64 Old Mint Street, Valletta

CANCER OF THE BREAST — A LOCAL STUDY

Saviour Pisani, Victor E. Sammut, Rose Galea

Cancer of the breast has, since classical times, been recognized as one of the most important of clinical affections. The superficial position of the tumour makes it one of the few growths which can be easily diagnosed. In fact, the generic name of all malignant tumours, the word 'cancer', stems from the similarity which the ancient investigators noticed between the infiltrative growth in the breast and a crab. The importance of this condition may perhaps be better appreciated from the fact that nearly 8,000 women developed breast cancer in 1962 in the British Isles.

The following is a report on what, we hope, is a representative section of the patients who present each year with the disease. The histories of 107 such patients were studied. Some of them were taken directly from the patients in the surgical wards, but the majority were taken from the records in the Surgical Outpatients Department and the Radiotherapy Department. 90% of our cases were treated in WSI in the period October 1963 to the present day.

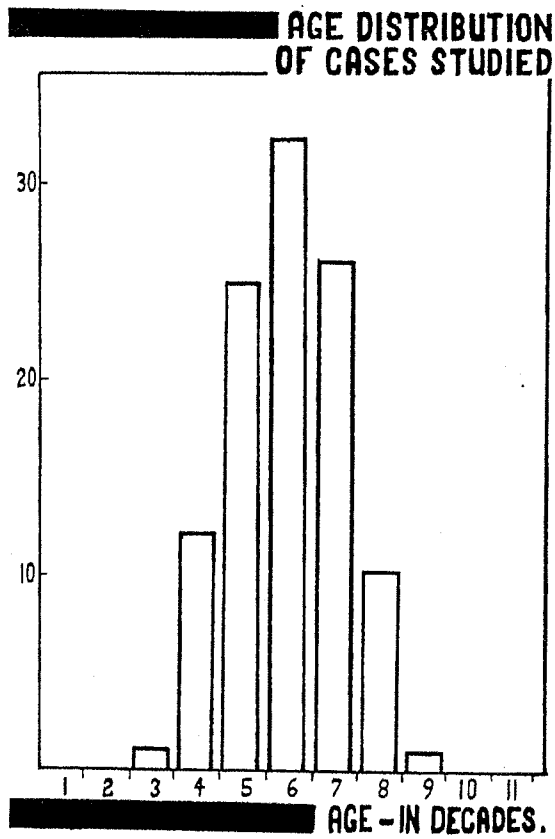


INCIDENCE OF BREAST CANCER IN MALTA 1958-66

INCIDENCE

Though the consultants and hospital staff remember some rare cases of breast cancer in males, we did not come across such cases in the histories we studied.

We tried to relate the incidence of the disease to the age of the patients when they first presented, and obtained very clear results. The incidence rose sharply from the third to the sixth decade, but it fell just as steeply after the latter age, as can be seen from the histogram.



The maximum incidence fell in the sixth decade, i.e. the age around the menopause. This finding may be a point in favour of the connection between cancer of the breast and the endocrine system—a topic which is still under discussion.

No definite relationship could be found between the incidence of the disease and the civil status. Of 107 cases, 69 were married, 29 unmarried, and 9 could not be determined. Unfortunately, we could not relate the incidence of the disease with the parity of the patients, owing to the lack of such information in the case-sheets.

PRESENTATION

The majority presented with a lump in the breast. In 64, the lump was on the left, and, in the remaining 43, it was on the right. However, in such a small survey, this curious disparity has no statistical importance. Pain was present in 30%—it was variably described as tender-

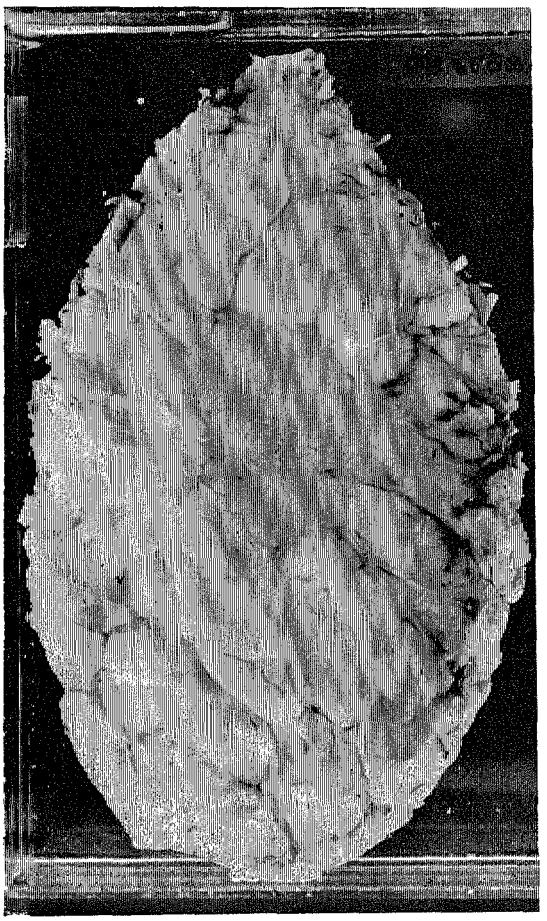


FIG. I

ness, tingling, heaviness, pricking sensations, burning and discomfort. The other 70% had no pain at all. 25% had retraction of the nipple. 10% had discharge—mucous, sanguinous, or purulent. A history of trauma to the breast preceded the symptoms in 12%..

A typical history was that of a 44-year-old patient who presented in October 1964 with a lump of 5 months' duration in the right breast. At the site there was pain, tingling and attachment of the lump to the surrounding tissues. The patient had not noticed any increase in size, and examination revealed no enlarged rerional lymph nodes. A biopsy of the lump was carried out and carcinoma simplex diagnosed. She was treated with radical mastectomy and radiotherapy, is being followed up as a surgical out-patient, and was lately described as being in a good state of health*. 3% of the patients we studied presented with Paget's disease of the nipple. They had ulceration and redness over the nipple accompanied by pain, itching, and discomfort. Most of them had been referred by their practitioners for 'eczema' and it was only in hospital that the correct diagnosis was made. A typical case was that of a married woman of 68 years, with 6 children, who presented in July 1964 with ulceration of the right nipple which had occurred 'on and off' for the previous 6 to 8 years, and discharge from the same site for the previous two years. On examination, Paget's disease of the nipple was diagnosed, and no metastases were found. Radical mastectomy was performed, and the patient, who was at last seen in January 1967, was reported 'well', with no evidence of recurrence.

UNUSUAL CASES

i) A 56-year-old lady was admitted to the medical wards with diarrhoea, vomiting, anorexia, and malaena since 10 days, and a provisional diagnosis of 'acute gastro-enteritis'. She died within a fortnight of admission. At postmortem a lump in the right breast and metastatic deposists in the liver were found. The cause of the malaena remained obscure. Microscopy confirmed the malignancy of the breast nodule.**

ii) A 50-year-old spinster presented in May 1966 with pain in her right hypochondrium. General examination revealed a hard lump in the left breast and enlarged lymph nodes in the left axilla, together with marked ascites. During laparatomy, after removal of a large amount of free fluid, the ovaries were found to be replaced by a large solid mass, and multiple secondary deposits were found in the omentum. The right ovarian mass was excised and sent for histology, which yielded the diagnosis of papillary carcinoma. After recovery from the operation she was discharged from hospital, but had to be readmitted in two months complaining of abdominal pain and exhibiting marked cachexia. Besides the lump in her left breast, hard lymph nodes could now be felt in the left axilla. Unfortunately, the history of the case gave us no further clue as to the fate of the patient.

iii) A 64-year-old patient went to her general practitioner in June 1966, and during the course of a general examination, he discovered a lump in her right breast about which she had previously known nothing at all. He referred her to hospital, where she underwent radical mastectomy and radiotherapy. Microscopical examination of the breast showed an extraductal infiltrative carcinoma superimposed on fibroadenosis, with no metastatis in the axillary lymph nodes.

*Section of tumour is seen in Figure 1.

**Section of tumour and liver metastases are seen in Fig. II.

FIG. II

PRECANCEROUS CONDITIONS

These were present in 20% of our cases: 10% had fibroadenosis, 8% had fibroadenoma, and 2% had cystic hyperplasia, in the same breast that contained the cancer. (It is noteworthy that fibroadenoma is not usually regarded as a precancerous condition per se, but we decided to mention it under this heading because in our cases it was associated with an active malignant growth in the same breast).

METASTASIS AND RECURRENCE

75% of our cases showed up with metastasis in three years' time. The sites of metatasis which we encountered are given in the following table:

Site of Metastasis	Number of Cases
Axillary lymph nodes	60
Bones (mainly ribs, vertebrae, pelvis)	11
Supraclavicular lymph nodes	5
Liver	4
Brain	5
Submandibular lymph nodes	2
Lungs	2
Ovary	2
Contralateral breast	2
Mesenteric lymph nodes	1
Colon	1
No metastasis	25
Recurrence in operation scar	7

An important finding was that only about 25% of the cases presented without metastasis, and it was these patients who had the best prognosis. The fact that about 75% presented with metastasis shows that, though the patients are aware of the potential dangers of a lump in the breast, they are not presenting themselves early enough. Perhaps it is salutary to point out that not all lumps in the breast are malignant, and anyhow, by avoiding her doctor, the patient is in no way avoiding her disease.

CASES OF METASTASIS AND RECURRENCE

i) A 55-year-old woman, single, was admitted to the surgical ward in January 1966 with intestinal obstruction. Six years previously she had had a radical mastectomy and bilateral oophorectomy for carcinoma in the right breast. At laparotomy the caecum, appendix, and ascending colon were found to be involved by malignant growth. A colectomy was performed, but the patient died in July 1966, three days after having developed jaundice.

ii) A 44-year-old patient presented in May 1966 with a painless ulcerated lump in her left breast, which had been there for two years. Following radical mastectomy and biopsy, the pathological diagnosis was that of anaplastic carcinoma proliferating within areas of fibroadenosis, with metastasis in the left axillary nodes. She was given a course of post-operative radiation at the Royal Marsden Hospital in July and was back in August 'feeling well'. However, later that month, she felt upset, and had erythema and swelling around the operation wound. In October pus was discharged from the operation site, and in November this site was reported as 'healing satisfactorily' but a large hard mass could now be felt in the right breast. Mastectomy and biopsy at this second site revealed a diffuse infiltrating carcinoma with foci of necrosis and diffuse metastasis in the right axillary lymph nodes. The patient was discharged in a satisfactory condition, but returned in January 1967 with a healed operation wound and palpable small nodules across the midline of the chest. Her condition continued to deteriorate, and she died in hospital in July 1967.

iii) In June 1964 a 36-year-old woman was admitted with a lump in her right breast which had appeared nine months previously and was increasing in size. The swelling was painful and tender. The pain radiated to the axilla and base of the neck. Malignancy was confirmed and she was treated with radiotherapy, bilateral oophorectomy, and endoxan. In February 1965,

she returned with recurrence in her right lung and ribs. In March, a lump was felt in her left breast, her left axillary lymph nodes pere palpable, and the supraclavicular lymph nodes were palpable on both sides. She died in May 1965.

CASES OF SPECIAL INTEREST

i) **Carcinoma of the breast complicating pregnancy:** This was the only case of this type that we found in our survey. The patient was a 36-year-old married woman with a history of eight full-term normal deliveries. In August 1964 she presented with a lump of six months' duration in her right breast. She was then in the seventh month of her ninth pregnancy. Her main complaint was pain in the right hypochondrium, felt since the previous six days, which increased on lying down. The findings on examination were: A large movable lump adherent to skin and muscles, Peau d'orange, no discharge, no enlarged lymph nodes, nipple retraction. A chest X-ray showed erosion of the posterior part of the right seventh rib, suggestive of metastasis. After a very short stay in the surgical ward, she was transferred to the antenatal ward, where an artificial rupture of membranes was performed, and an oxytocin drip was set up, in an attempt to induce labour. These attempts were unsuccessful, so she was taken to the operating theatre, where a live male foetus was delivered during a lower segment caesarean section accompanied by bilateral oophorectomy. Subsequent treatment consisted of testosterone deposits and endoxan. In December of the same year she complained of pain in the neck radiating to her right shoulder and arm, and severe right sided headache. Later she also had pain and paraesthesiae down her left arm. In October 1965 she was reported 'very ill in bed', and in November 1965 she died at home in severe dyspnoea and pain.

ii) **Lymphosarcoma:** A 54-year-old woman was admitted in June 1966 with a history of a painful lump of 7 weeks' duration in her left breast. The findings of note were retraction of the left nipple and redness of the surrounding skin. No lymph nodes were palpable. Biopsy of the lump enabled the diagnosis of lymphosarcoma to be made. She was treated by radiation and endoxan. In May 1966 lymph nodes became palpable in the left axilla. These were found to respond favourably to endoxan. In October 1967 X-rays showed shadows in both femora and in the pubic bone strongly suggesting metastases. She is still alive today, but is seriously ill.

TREATMENT AND PROGNOSIS

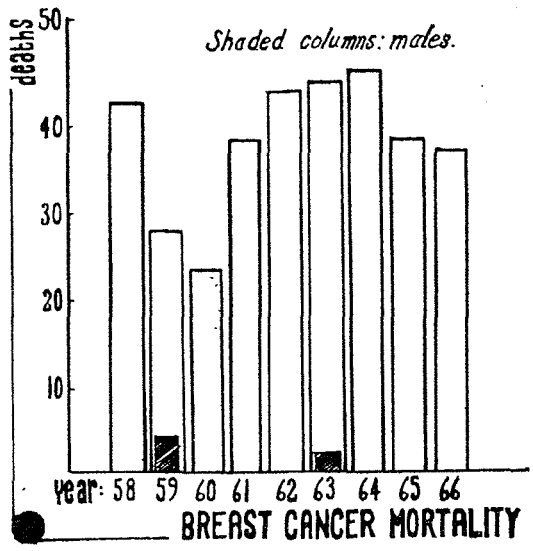
The treatment which our cases received can be summarised as follows:

A. Local Treatment
1. **Surgical** i) Partial mastectomy
   ii) Radical mastectomy
2. **Radiation**

B. Systematic Treatment
1. **Chemotherapy**
2. **Hormone Therapy**
3. **Sterilisation**
   i) Surgical
   ii) Radiation

Radical surgery gave the best results by far, especially when followwed up by a course of radiation. Endoxan and other cytostatics were given in the post-operative preradiation period. Some cases were also sterilised either surgically or by radiation, and follow-ups were given cytostatics and/or hormones, especially oestrogens and progesterone. They were monitored with serial X-rays and blood investigations.

Fifteen of our cases were notified as dead. About some of the rest we are not completely certain owing to the patients or their relatives not turning up regularly at the outpatients department.

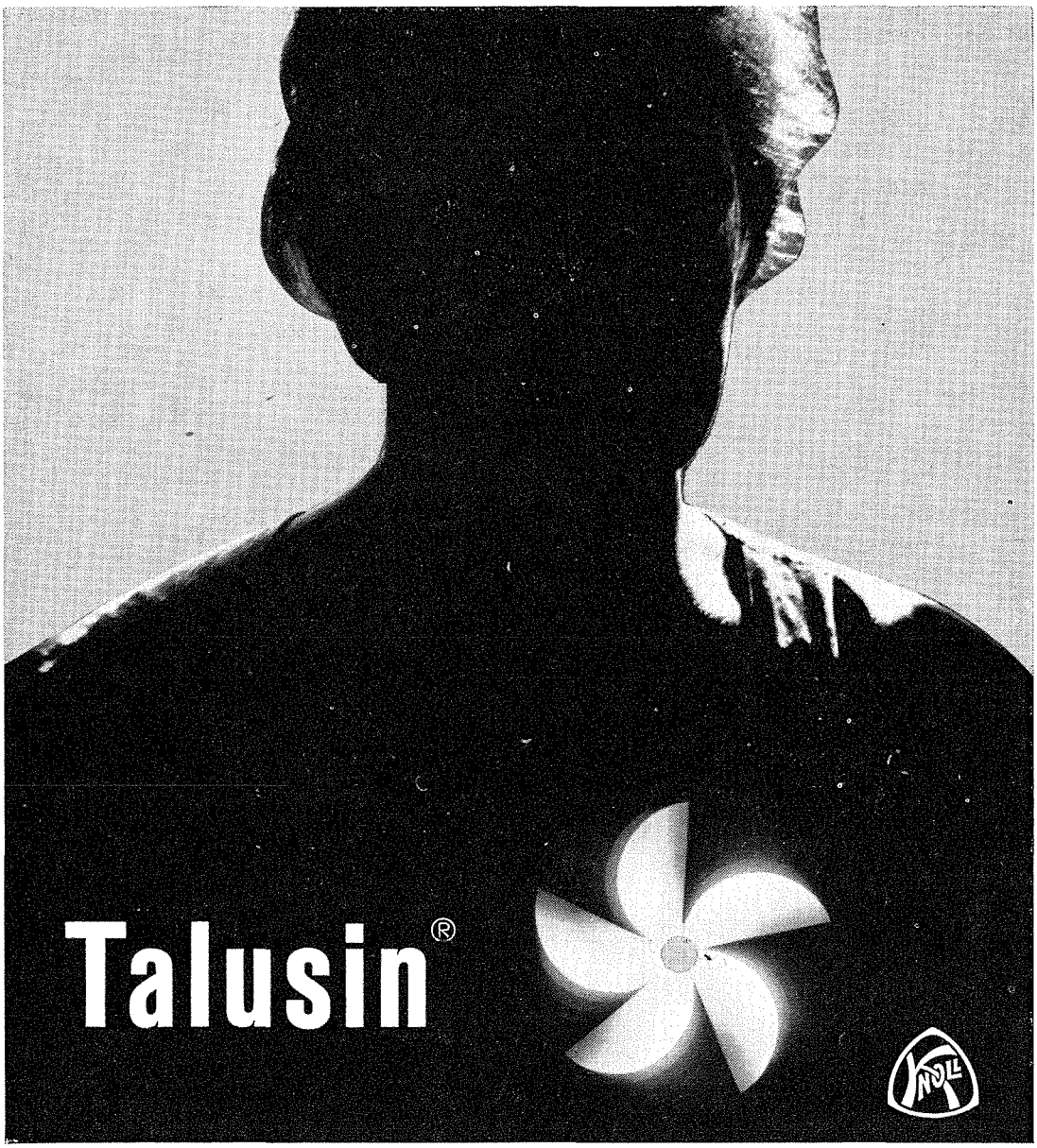


CONCLUSION

We conclude that cancer of the breast is no longer a killing condition if diagnosed and treated adequately and in time. Thus the value of a good general examination of a patient presenting with any signs and symptoms, even if apparently totally unconnected with cancer of the breast.

Finally we would like to thank all those who helped us collect material for this essay. We

thank especially the Dean and Prof. Xuereb, Dr. Calleja and Dr. Sultana and the sisters and nurses of the various wards and outpatients departments. We would also like to thank the C.G.M.O. for his interest and encouragement, and Mr. Serge of the Statistics branch of the Medical and Health Department for giving us the figures for incidence of, and mortality from, breast cancer in Malta, the Medical School photographer for his patient help, and the nameless housemen of the past years without whose history sheets such an essay would have been impossible.



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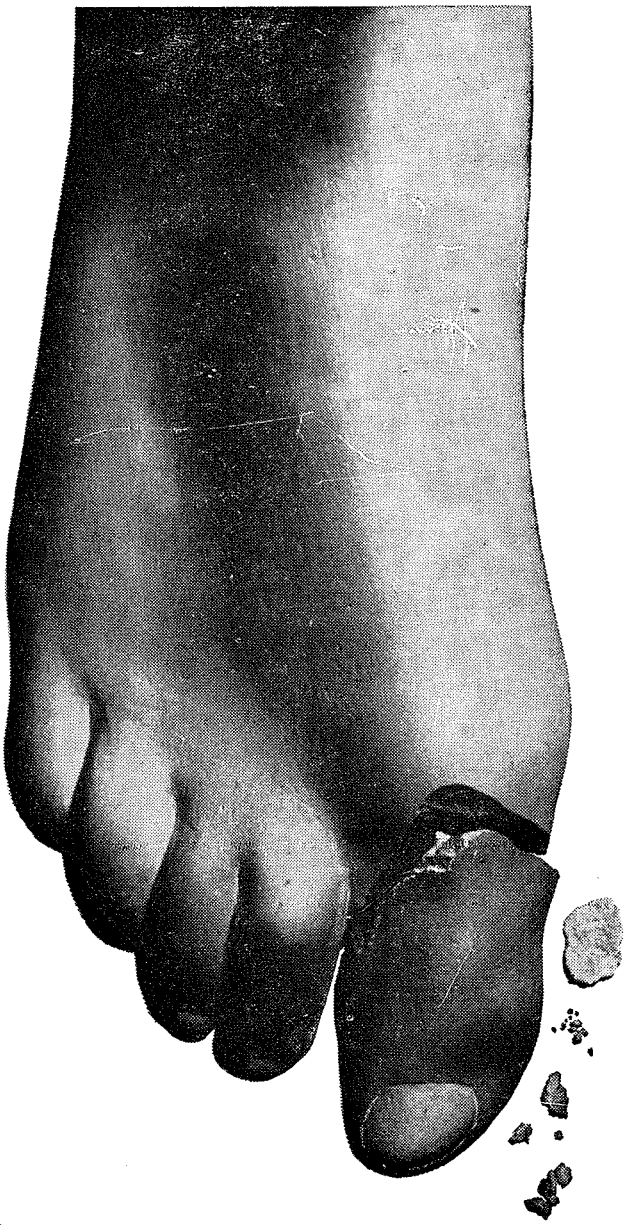

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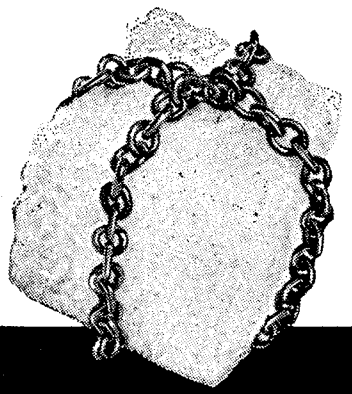

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